University of Alberta

The Role of Matrix Metalloproteinases in Tissue Remodeling in Sodium Metabisulphite and Cigarette Smoke Induced Epithelial Injury.

by

©

Jie Ding

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ABSTRACT

Matrix metalloproteinases (MMPs) are a diverse family of enzymes that mediate airway tissue remodeling and epithelial reactivity. Changes in the levels of MMPs and their inhibitors (TIMPs) may contribute to the pathophysiology of the injury induced by sodium metabisulphite (MB), which releases sulphur dioxide when dissolved in water, and cigarette smoke (CS). Frog airway epithelial tissue injured by MB (10^{-1} M) was found to show significantly more MMP-9 activity compared normal tissue, suggesting that remodeling of activated airway epithelial cells may be due, in part, to altered levels of MMPs. In airway epithelia injured by sodium metabisulphite, significantly less protein with molecular weight consistent with TIMP-1 compared to normal epithelia was observed, and pre-incubation in *Radix Ophiopogonis* (ophiopogon root, OP) extract, was found to have significant effects on decreasing the MMP-9 activity and increasing the TIMP-1-like protein level.

The study of agents modifying mucociliary clearance showed that MCT (mucociliary clearance time) was significantly prolonged after injury with MB (10^{-1}M), and this prolongation could be significantly decreased by pre-incubation with OP extract. These findings suggest that MMPs play an important role in tissue remodeling of the injury induced by MB, and OP may have effects on ameliorating or reverting this injury.

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ABBREVIATIONS AND UNITS

The following abbreviations, definitions and units have been used throughout this thesis.

°C	degrees Celsius
ANOVA	analysis of variance
BAL	bronchoalveolar lavage
BH_4	tetrahydrobiopterin
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CBF	cilia beat frequency
cm	centimetre(s)
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
CS	cigarette smoke
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthase
Et al	*et alii* (Latin, "and others")
EtOH	ethanol
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide

FR	frog Ringer
g	gram(s)
Gly	glycine (amino acid)
GPCR	G protein-coupled receptors
h	hour(s)
H_2O	water
H_2O_2	hydrogen peroxide
HCL	hydrochloric
HNE	human neutrophil elastase
HOCL	hypochlorous acid
i.e.	*id est* (Latin, "that is")
IFN	interferon
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
IgG	non-special mouse immnoglobulin G
KCL	potassium chloride
kDa	kilodalton
l	liter(s)
L-NAME	N^G-Nitro-L-arginine methyl ester hydrochloride
LPS	lipopolysaccharide
LTB-4	leukotriene B_4
m	meter(s)

mm	millimeter(s)
M	moles.l^{-1}
MB	sodium metabisulphite
MCP	monocyte chemoattractant protein
MCT	mucociliary clearance time
MCR	mucociliary clearance
MCV	mucociliary clearance velocity
$MgCl_2$	magnesium chloride
min	minutes(s)
MMP	matrix metalloproteinase
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MT-MMP	membrane type matrix metalloproteinase
n	number of animals
N_2	nitrogen
NaCl	sodium chloride
NaN_3	sodium azide
NEBs	neuroepithelial bodies
NF-κB	transcription factors nuclear factor kappa B
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase

O_2	molecular oxygen
OD	opical density
ONOO-	peroxynitrite
OP	*Radix Ophiopogonis* (ophiopogon root)
p value	probability (of incorrectly rejecting the null hypothesis)
PAF	platelet activating factor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	logarithmic unit measuring acidity
PSI	pollution standard index
RANTES	regulated on activated, normal T expressed and secreted protein
RNA	ribonucleic acid
RNS	reactive nitrogen species
s	second(s)
SD	standard deviation of the mean
SDS	sodium dodecyl sulphate
SE	standard error
SEM	scanning electron microscope
SO_2	sulphur dioxide
TGF-β	transforming growth factor-β

TIMP	tissue inhibitior of metalloproteinases
TNF-α	tumour necrosis factor-α
TPBS	phosphate buffered saline with Tween
Tris-Cl	tris[hydroxymethyl]-amino methane hydrochloride
WHO	The World Health Organization
CO	carbon monoxide
NO_2	nitric dioxide
VCAM-1	vascular cell adhesion molecular-1

CHAPTER 1. RATIONALE, HYPOTHESIS AND LITERATURE REVIEW.

1.1 RATIONALE

Air pollution and cigarette smoke contribute to lung diseases, including respiratory tract infections, chronic bronchitis, emphysema, asthma and lung cancer (1, 2, 3, 4). Sulphur dioxide (SO_2) is one of the most important components of the air pollution (5). Sodium metabisulphite (MB), dissolved in water, releases small quantities of SO_2 of comparable concentrations to those found in polluted air.

Topical application of MB may cause epithelial injury to frog palate tissue (7). The MB injury is similar to that induced by exposure to cigarette smoke (CS) in the same tissue (8).

The process that regulates the injured tissue remodeling is believed to involve matrix metalloproteinases (MMPs). MMPs are a group of extracellular matrix (ECM)-remodeling enzymes that play a central role in epithelial tissue development, and are regulated, in part, by tissue inhibitors of metalloproteinases (TIMPs) (6).

Mucociliary clearance, a vital mechanism of pulmonary defence, requires the coordination of many parameters for its effectiveness; these include cilia beat frequency (CBF), mucus secretion rate, average depth of mucus layer, mucus rheology, and the inherent transportability of the mucus (9, 10). Progressive injury of the epithelium leads to overall impairment of mucociliary clearance.

In previous investigations using a pathologic quail model, *Radix Ophiopogonis* (OP) was demonstrated to significantly revert (11) the human neutrophil elastase (HNE)-induced increase in mucociliary clearance time (MCT), a directly measurable parameter of mucociliary clearance, but its effects on MB-induced epithelial injury and the activation of MMP-9 and release of TIMP-1 were not studied. Therefore, we examined the mucociliary clearance time and the activation of MMP-9 and release of TIMP-1 (as indicators of tissue remodeling) from injured epithelial tissue and OP pre-incubated injured epithelial tissue.

Furthermore, nitric oxide (NO) has recently been found to be involved in inducing alveolar epithelial injury in the mouse model (12); however, its role in normal and MB-injured airway epithelia has not been examined. Therefore, we examined NO as the mediator of one of the possible pathways through which MB works on the ciliated epithelium. We pre-treated the frog palate with an NO inhibitor, L-NAME, prior to the application of MB; then we investigated the resulting of MCT and MMP-9 alteration compared with normal tissue and MB (10^{-1}M) treated tissue.

1.2 HYPOTHESES

As mentioned in the rationale, MMPs play an important role in ECM-remodeling and can modulate airway epithelial reactivity and mucociliary clearance, which are vital pulmonary defences, very sensitive to airway injuries. I will be describing four separate projects in this thesis, examining the role of MMPs, mucociliary transport and NO in the frog palate epithelial injury induced by MB. My general hypotheses are that alterations in the activation of MMPs and release of TIMPs may contribute to the pathophysiology of the frog palate epithelial injury induced by MB and CS, and furthermore OP may partially revert the MB-induced injury.

My specific hypotheses and experimental approach are described below:

Hypothesis 1: MCT is elevated in MB (10^{-1}M) injured frog palate tissue and pre-incubation of palates in OP extract (1g/ml) prior to the MB treatment will reduce MCT close to baseline values. *Experimental approach 1:* Bullfrog palates pre-incubated in FR are topically treated with FR or MB, or pre-incubated in OP followed by FR or MB treatment, respectively. The level of MCT is assessed by recording the time for the displacement of a drop of mucus over a 5mm pathway along the ciliated surface of the frog palate.

Hypothesis 2: The activation of MMP-9 is increased and/or the release of TIMP-1 is decreased in MB (10^{-1}M) injured frog palate tissue; this imbalance in protease/ antiprotease levels can contribute, in part, to the observed tissue injury; and pre-incubation of palates in OP extract (1g/ml) prior to the MB treatment will decrease the activation of MMP-9 and/or increase the release of TIMP-1. *Experimental approach 2:* Tissue samples from healthy/normal bullfrog palates, normal frog Ringer (FR) pre-incubated and MB treated palates, and OP pre-incubated and MB treated palates are collected. The levels of MMP-9 and TIMP-1 are assessed by zymography and Western blot analysis, respectively.

Hypothesis 3: The activation of MMP-9 is increased and mucociliary clearance is impaired in cigarette smoke-exposed frog palate epithelial tissue, which relates to the damage to the epithelium. *Experimental approach 3:* Bullfrog palates are divided in half and kept under identical conditions. One half is exposed to smoke from up to four cigarettes and one is not (14). The level of MCT is recorded and tissue samples are collected on each palate. The levels of MMP-9 are assessed by zymography.

Hypothesis 4: Nitric oxide may play a role in the epithelial injury induced by MB. *Experimental approach 4:* Bullfrog palates are topically treated with normal FR or MB (10^{-1}M), or pre-treated with a nitric oxide synthase inhibitor: N (omega)-nitro-L-arginine methyl ester (L-NAME) (10^{-4}M) (15) followed by MB (10^{-1}M) treatment. The level of MCT is recorded and tissue samples are collected on each palate. The levels of MMP-9 are assessed by zymography.

I will begin this thesis by describing the formation of the injury in respiratory diseases, followed by how MB and CS may injure frog palate epithelium. I will start with the physiological changes take place in the epithelium, which also lead to the alteration of the MCT. There will be a special emphasis on the critical role of MMPs in ongoing tissue remodeling within frog palate epithelium and how alterations in the levels of these enzymes and may contribute to altered epithelium reactivity. Meanwhile, the effect of OP on MB-injured frog palate epithelium will also be addressed, with a discussion on the regulation of MMPs and their inhibitor by OP. Finally I will examine the possible role NO may play in the epithelial injury induced by MB.

1.3 AIRWAY INJURY IN RESPIRATORY DISEASE

The respiratory system is vital to sustain life. It is the body system responsible for transport of gas - namely oxygen - to the body for exchange with CO_2. The appropriate development and condition of the airways is essential for a healthy respiratory system. The airways consist of the entire pathway for airflow from the mouth or nose down to the alveolar sacs. Smoking (16), occupational exposure (17), air pollution (18), infection (19) and allergens (20) may cause airway injury, and lead to acute or chronic respiratory disease or dysfunction.

The airways are the physical pathway for the tidal flow and bulk mixing of alveolar and atmospheric gases. The airway has a number of complex functions, not all of them directly involved in respiration. For example, the mouth and pharynx are also functional parts of the digestive system, and they work together with the vocal cords and the thorax to produce phonation. These structures also have the ability to prevent foreign material from entering the lower airway.

Generally, the airways can be specified as conducting and respiratory portions. The conducting potions, which consist of a series of air passages, can be divided anatomically into the upper respiratory tract and the lower respiratory tract. The upper respiratory tract consists of the nasal cavity, paranasal sinuses and nasopharynx, and ends at the larynx. The larynx acts along with the epiglottis to guard the entrance to the trachea and lower airways. It also functions as the organ of voice. The lower respiratory tract begins with the trachea, which enters the thoracic cavity and subsequently divides into two main bronchi, one supplying each lung. The bronchi then divide repeatedly, forming airways of ever-decreasing diameter. The smallest bronchi are called terminal bronchioles; these are the last of the purely conducting portion of the lungs. Each type of airway has its own characteristic structural features. With decreasing diameter of the airways, structural changes are gradual rather than abrupt transitions. The respiratory portions of the lungs are the structures that are directly involved in the transfer of oxygen from the air into the blood and carbon dioxide from the blood into the air. Although the respiratory portions perform important functions and are involved in a number of disorders, it is the conducting portion that most of the respiratory diseases have their origins.

The respiratory epithelium is the characteristic epithelium of the upper respiratory tract, trachea and large bronchi. It is typified by a pseudostratified columnar, ciliated epithelium with mucus-secreting goblet cells. When airways are exposed to smoke, occupational exposure, polluted air, infection or various allergens, injury to the airway epithelia will take place. The initial cellular and exudative phase is characterized by an influx of neutrophils, elaboration of oxygen-free radicals, liberation of complement degradation products, and production of inflammatory mediators. Inflammation will arise, accompanied by bronchial hyperresponsiveness, mucus hypersecretion, submucosal hypertrophy, epithelial cell shedding (21), fibrosis, inappropriate remodeling, and reduced pulmonary function, which are the initiations of most acute or chronic respiratory diseases. Airway remodeling is an alteration in size, mass or number of airway tissue structural components that occurs during growth or in response to injury and/or inflammation. Inappropriate airway remodeling impacts negatively in the maintenance of normal airway function (11, 21). Airway injury is taken as one of the most important early signs of respiratory disease.

1.4 AIRWAY INJURY INDUCED BY SODIUM METABISULPHITE AND CIGARETTE SMOKE

Over the last decade, the death rate for lung disease has risen faster than that of any of the top five causes of death; smoking and air pollution remain the major causes. Smoking and air pollution contribute to respiratory tract infections, asthma, and lung cancer. Sulphur dioxide (SO_2) is one of the most important components of air pollution. Major health concerns associated with exposure to high concentrations of SO_2 include effects on breathing, respiratory illness, alterations in pulmonary defences, and aggravation of existing cardiovascular disease (22).

It has been reported that airway epithelial damage could be observed in dog models after inhalation of SO_2 for as little as 1 h (141). A wide spectrum of mucosal cell injury during the response phase was observed. Individual cells, rows of mucosal cells and entire regions were exfoliated (23). Changes in airway responsiveness induced in rats by repeated exposure to SO_2 gas have also been found. With daily exposure to high concentrations of SO_2, there is chronic injury and repair of epithelial cells. Over time, rats develop mucus hypersecretion, airway inflammation, increased airway resistance and airway hyperresponsiveness (24). Once the airway has been injured by high-concentration SO_2 even for a short period of time, the damage to the airway tissue may be non-restorable (30). The effects of exposure to SO_2 gas can be duplicated by exposure to sodium metabisulphite. Sodium metabisulphite ($Na_2S_2O_5$, **Figure 1.2**) is a white, or off-white, finely crystalline powder with a faint sulphur dioxide odor, which can be dissolved in water, releasing sulphur dioxide. $Na_2S_2O_5$ has been employed in airway injury models to study mucus hypersecretion and hyperplasia (25).

Cigarette smoke (CS) is the main cause of chronic obstructive pulmonary disease (COPD) (26). CS can activate macrophages and airway epithelial cells in the respiratory tract. Macrophages and epithelial cells release neutrophil chemotactic factors (interleukin-8 (IL-8), leukotriene B_4 (LTB-4), which can recruit neutrophils to the inflammatory site. In turn, neutrophils, and macrophages release proteases (neutrophils release elastases, cathepsins, and MMPs; macrophages release MMPs). These proteases can break down the connective tissues in both lung parenchyma and airway epithelium and lead to a variety of respiratory pathologies (27). It is proposed that cigarette smoke destroys the protease/antiprotease balance and leads to the epithelial injury and inappropriate remodeling (28). However, recently more and more evidence showed that this injury of epithelia is more likely to be an acute procedure, which may involve the activation of MMPs (29).

1.5 MUCOCILIARY CLEARANCE MECHANISM

The mucociliary clearance system is one of the lung's primary defence mechanisms. It protects the conducting airways by trapping and sweeping away bacteria, inhaled particles, and cellular debris. Disruption of normal secretion or mucociliary clearance impairs pulmonary function and lung defence and increases risk of infection (73). The system probably also serves as a reservoir of humidity for incoming air, and it may help to modify the airway response to inhaled agents (32).

The airway epithelium consists of three strata. The surface stratum is composed largely of ciliated columnar cells, which are interspersed with non-ciliated, microvillus cells and goblet cells. The cilia, whose structure is remarkably constant throughout the animal kingdom, beat in an asymmetric pattern, with a fast forward stroke during which the cilia are stiff and outstretched, and a slower return stroke, during which the cilia are flexed. The direction of the forward stroke, and thus of fluid movement, is predominantly cephalad. The fluid lining the epithelium consists of two layers: the lower, a non-viscid serous fluid in which the cilia beat, and the upper, a visco-elastic material (the mucus), which lies on the top of and is propelled by the cilia. Mucus is secreted by both goblet cells and subepithelial glands. Mucus transport is theoretically possible whether the cilia contact the mucus or not; however, the exact nature of the relationship between the properties of mucus and mucociliary clearance (MCR) would likely depend on the specific interactions that occur between the cilia and the mucus layer (32).

Mucociliary clearance requires the coordination of mainly three kinds of factors: ciliary serous fluid and mucus factors (76). Ciliary factors that affect MCR are mainly ciliary amplitude and beat frequency, which together determine the maximal velocity at the tips of the cilia, and hence the maximal forward velocity of the mucus layer. In principle, the faster the cilia beat, the higher the MCR. Also, longer cilia should be able to clear mucus faster because they can generate greater forward velocities. The density or spacing between cilia will also affect MCR, because the greater the distance between the cilia, the more energy will be dissipated in the mucus, reducing the net forward velocity. Serous factors that affect MCR include serous fluid viscosity and serous fluid depth. If the serous fluid is too viscous, the cilia will not be able to move very well within it, and the decreased ciliary tip velocity will lead to a reduction of MCR. If the serous fluid is too deep or too shallow, MCR will also decrease.

Mucus factors affecting MCR are the mucus depth and mucus visco-elastic properties (10, 34). Mucus needs to be both viscous and elastic. The elasticity of mucus is important for clearance by cilia because it efficiently transmits energy without energy loss. The viscosity of mucus results in energy loss, but it is necessary so that mucus can be extruded from submucosal glands, and displaced and either expectorated or swallowed. A balance between these factors must be maintained for optimal MCR. The transport velocity of mucus is directly related to mucus elasticity and the depth of the periciliary fluid, and it is inversely related to mucus viscosity (32). An ideal visco-elastic ratio may exist for optimal mucociliary interaction; an increase in viscosity and/or a decrease in elasticity would result in a reduced transport rate (35).

Mucociliary clearance time (MCT) is one of the commonly used measurements of the epithelial clearance function. It is the time recorded by measuring the rate of displacement of a certain distance with a certain mucociliary clearance velocity (MCV) (76). However, because of the difficulties in studying mucociliary clearance in intact mammalian airways, investigators long ago turned to more easily accessible systems in which to study the principles that regulate mucociliary transport. The frog palate was perhaps the most obvious choice because of its ready accessibility and its many similarities with higher systems. The frog palate has a pseudostratified epithelium (**Figure 1.1**) composed of both ciliated and mucus-secreting cells and covered with a two-layered periciliary film. Since this is quite similar to the situation in human conductive airways, the frog palate has long been considered a useful model to study mucociliary clearance related to human lung disease (76). The first description of the weight-carrying capacity of frog palate cilia was published by Stewart in 1948 (36). Since then, there have been a large number of studies carried out (37, 38, 39), which provide the basis for the use of the excised, mucus-depleted frog palate as a practical model for testing the inherent clearability of mucus collected from various sources.

1.6 MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are a group of extracellular matrix (ECM)-remodeling enzymes that may play a role in airway remodeling (72); and altered levels of these enzymes and their inhibitors may contribute to the inappropriate remodeling after the injury. This section will provide an overview of MMPs and their inhibitors, with a special emphasis on their roles in airway epithelia remodeling after injury.

1.6.1 Overview

MMPs are a family of enzymes (**Table 1.1**) involved in remodeling of the extracellular matrix (ECM) in a number of physiological and pathophysiological processes. In normal physiology, MMP activity is associated with angiogenesis (40), wound healing (41), remodeling of bone (42), neutrophil function (43) and macrophage function (44). On the other hand, unregulated MMP activity has been implicated in cancer invasion (45), rheumatoid arthritis and osteoarthritis (46), fibrotic lung disease (48) and degradation of the myelin-basic protein in neuroinflammatory disease (47).

MMPs are secreted as partially active zymogens that require proteolytic cleavage (49, 50) or reconfiguration (by biological molecules *in vivo*) (51, 52, 53) of the N-terminal pro-peptide for full activation. The zymogens (pro-MMPs) consist of three discernible domains: the autoinhibitory domain, the zinc-binding catalytic domain, and the hemopexin-like C-terminal domain (50). The amino-acid sequence of PRCGVPD in the autoinhibitory domain is thought to be involved in maintaining the inactive form of the enzyme by coordinated binding of cysteine thiolate and zinc ion, which protects the active site from substrates (54). Once the cysteine-zinc interaction is interrupted, catalytic activity is restored. This is known as the cysteine switch activation mechanism. MMPs are active at neutral pH and require calcium (Ca^{2+}) for full activity (33). Pertinent to our studies, MMP-9 is the gelatinase produced by many cell types including epithelial cells (55), neutrophils (56) and has the ability to degrade basement membrane collagens (57). When MMPs are released into the extracellular environment, or when they are membrane-bound and in contact with the pericellular zone, these enzymes degrade a broad range of connective tissue proteins, and are important during the development of healthy tissue as well as in remodeling of damaged tissue (58).

The regulation of MMPs is complex and occurs at multiple levels including gene activation and transcription, translation and secretion of the latent enzyme, proenzyme activation and inactivation by endogenous inhibitors (54). *In vivo*, MMP activity is regulated by tissue inhibitors of metalloproteinases (TIMPs), which interact with the proenzyme at the amino terminus, the region flanking the enzyme active site (59, 60). The resulting steric hindrance effectively blocks the MMP active site from binding to components of the ECM. Under conditions of stasis, there is an equimolar ratio of MMPs and TIMPs outside the cell, with one TIMP binding to one MMP, resulting in a lack of proteolytic activity and stable cell populations (61). During cell proliferation and migration, however, there must either be a shift in the relative levels of MMPs and TIMPs or a dissociation of TIMPs from MMPs. TIMP-1 inhibits the active form of all

MMPs and the latent form of MMP-9 (pro-MMP-9) and is the most widely distributed TIMP (62) in the animal body. In most cells, MMP-9 is secreted as a complex with TIMP-1. The function of the interaction is not known. TIMP-2 binds to both the inactive and active forms of MMP-2, while its inhibitory effect over the other MMPs is significantly lower (148). TIMP-2 also mediates the activation of pro-MMP-2 through the formation of a trimolecular complex between membrane-type MMP (MT-MMP), TIMP-2 and pro-MMP-2. TIMP-3 which can inhibit MMP-2 and MMP-9 (149), is localized to the ECM in both its glycosylated and unglycosylated forms (150). Its mRNA species are constitutively expressed by human chondrocytes (151). TIMP-4 is the most neutral TIMP protein under physiological conditions (pH 7.4) (142) and has been detected in human cartilage (91). TIMP-4 is a good inhibitor for all classes of MMPs without remarkable preference for inhibiting specific MMPs (77). It regulates MMP-2 activity both by inhibiting MT1-MMP and by inhibiting activated MMP-2 (82, 145).

1.6.2 The importance of MMPs in airway epithelial injury

MMPs are vital during airway remodeling, a process which involves an alteration in size, mass or number of tissue structural components that occur during airway growth or in response to airway injury and/or inflammation (63). Investigators have demonstrated that MMP-9 has specific affinity for the subepithelial basal lamina, a specialized nonfibrillar connective tissue structure that anchors epithelial cells to parenchymal surfaces, and it may contribute to the basement membrane injury in acute allergic airway inflammation (64). The processes that are involved in airway epithelial remodeling likely involve

MMPs and their ability to remodel ECM. Indeed, MMP-9 can be expressed in many inflammatory cells, such as macrophages (65), lymphocytes (66), mast cells, or eosinophils (67) of which all are involved in asthma. It has recently been demonstrated that human bronchial epithelial cells are able to constitutively produce MMP-9 and upregulate its production in response to inflammatory injury (68, 69), thus playing a role in the physio-pathological remodeling of airways. More precisely, the excessive release of this enzyme not counterbalanced by specific inhibitor TIMP-1 in expectorations from children with cystic fibrosis was hypothesized as a mechanism to initiate airway damage (70, 71).

1.7 EFFECTS OF SODIUM METABISULPHITE ON AIRWAY INJURY

There are many ways to induce airway epithelial injury. As one of the most important components of air pollution, SO_2-induced airway injury has always attracted the attention of the researchers. This section will describe sodium metabisulphite (MB), which releases SO_2 when it meets water, in the context of induced airway injury, models of SO_2 toxicosis, and the regulation of MMPs and TIMPs.

1.7.1 SO_2 levels in polluted air

Air pollution is a major environmental health problem globally. The World Health Organization (WHO) considers that air pollution is damaging the resources needed for the long-term sustainable development of the planet (74). Air pollution degrades the environment, reduces visibility, and produces serious health effects. Air pollutants are usually classified into suspended particulate matter (dusts, fumes, mists, smokes), gaseous pollutants (gases and vapours) and odours.

Suspended particulate matter consists of finely divided small particulates including inorganic and organic carbon, acidic or neutral sulphates and nitrates, fine soil dust, residues of lead and other metals, asbestos and other fibres. Gaseous pollutants mainly comprise sulphur dioxide (SO_2), oxides of nitrogen (NO_x), photo-oxidants and carbon monoxide (CO). SO_2 is released into the atmosphere primarily as a result of industrial combustion of coal and oil. A smaller proportion is produced by vehicular sources (diesel cars, buses, and trucks) due to sulphur contained in the fuel (75).

Various local and regional governments now publicize a "pollution standard index," or PSI to warn patients to stay indoors when the air is unsafe (**Table 1.2**). The PSI scale ranges from 0 to 500 and is based on the air pollutant with the highest concentration at the time the test is done. Five major pollutants are measured at various points throughout the area: nitrogen dioxide (NO_2), SO_2, CO, photochemical oxidants (mainly ozone), and particulate matter. The PSI was originally developed by the U. S. Environmental Protection Agency to provide consistency in reporting on air quality. The PSI is published every morning in large city newspapers or is available from your local pollution control agency. Generally, when the concentration of SO_2 in the ambient air is higher than 0.3 ppm, it is unhealthy to humans and will be considered as polluted air.

1.7.2 Models of airway injury

It has been known for some time that particle clearance in the airway is dependent on mucus and cilia (76). The study of mucociliary clearance in the intact mammalian airway is technically difficult. As a result, the frog palate model has been used extensively because of its ease of preparation and its structural similarities to the human conductive airways. The frog models have been employed in two main ways in the past. First, fresh palates, which employ endogenous mucus produced and secreted from the palate epithelial goblet cells, have been used to study cilia function and mucus transport parameters. Secondly, the mucus-deleted frog palate model has been used to test exogenous mucus samples (such as those from cystic fibrosis patients) for evaluation as mucociliary agents (14). In order to investigate the damage to the epithelium and evaluate the changes in the mucociliary clearance function, we used the first approach to set up our model in our studies.

It has been reported that long-term exposure of dog airway to SO_2 will induce airway epithelial injury and features of chronic bronchitis. The concentration of SO_2 that has been used experimentally to model a polluted air environment was as high as 500 ppm (140). However, it is not clearly known how these acute, high concentrations of SO_2, relate to the more modest occupational exposures, in terms of airway physiology and mucociliary clearance. To create an injury model from the fresh frog palate, we applied a topical solution of sodium metabisulphite (MB) (up to 10^{-1}M) to the palate surface; consistent temperature and humidity were maintained. MB, topically applied, releases sulphur dioxide (SO_2) *in situ*, producing similar lesions to classic SO_2-induced bronchitis (14). This agent has been used as an aerosol in other airway epithelial models such as guinea pig (25) and rat (7) to study hypersecretion and hyperplasia. After calculation, we found that the SO_2 generated in our experiment from MB (10^{-1}M) was no more than 2 ppm (the exact amount depends on how much MB was applied), which is more than twice the concentration of the concentration of SO_2 in pollution standard index (PSI) 500 (very hazardous polluted air); but still much less than the concentration applied to a experimental dog model of a polluted air environment (as high as 500ppm) (140). The MB (10^{-1}M) solution was demonstrated to produce significant effects to MCT on the palate surface (increase MCT by more than 50%). Minimal effects on mucociliary clearance were noted on the frog palate with MB (10^{-2}M), however we were more interested in those damages which are more acute and more significant, thus we use MB (10^{-1}M) as our principal application dose.

1.7.3 Mechanism of sodium metabisulphite induced injury

In our study, MB was chosen as an agent to perturb normal mucociliary activity on the palate because of its reported action to release SO_2 *in situ* (14). As will be shown in Chapter 2, there is an acute effect on mucus transport, which occurred rapidly after the application of MB (10^{-1} M) to the frog palate. Mucociliary clearance function seemed to be seriously impaired. The cause of the acute effect is unclear, although pH changes and/or oxidants may be possible factors. It has been found recently that pH may play a role in the process that epidermal growth factor (EGF) promotes gastric mucosal restitution by activating Na^+/H^+ exchange of epithelial cells (78). MB (10^{-1} M) with a pH of 5.5, when applied to the palate surface, reduced the palate epithelial pH by 0.5 pH units to 6.4. The time frame and the magnitude of the pH change may have been adequate to influence ion channels and stimulate the release or upregulation of inflammatory mediators (7). At the same time, in an *in vitro* system, it was demonstrated (79) that the ciliary beat frequency of bronchial cells was stable between pH 7.5 and 10.5. At lower pH values, ciliary beat frequency decreased significantly.

Nitric oxide, a potentially toxic molecule, is formed endogenously in the human lung and is implicated in a wide range of biological functions (80). Lung cells capable of producing NO include macrophages, neutrophils, granulocytes, endothelium, fibroblasts, vascular smooth muscle cells, mast cells and epithelial cells, including type II alveolar cells (81). Since its discovery as a biological messenger molecule more than 10 years ago, the gaseous molecule nitric oxide (NO) is now well recognized for its involvement in diverse biological processes, including vasodilation, bronchodilation, neurotransmission, tumor surveillance, antimicrobial defence and regulation of inflammatory-immune processes (80, 83, 84). In the respiratory tract, NO is generated enzymically by NO synthase (NOS), that are present to different extents in numerous cell types, including airway and alveolar epithelial cells, neuronal cells, macrophages, neutrophils, mast cells, and endothelial and smooth-muscle cells. Normally, NOS has three isoforms: eNOS, nNOS, and iNOS. eNOS is a membrane bound protein due to a myristylation tether. E-NOS and nNOS contain flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (BH_4) tightly bound to the enzyme. When cells are activated intracellular calcium is increased which binds to calmodulin and activates it. The calcium/calmodulin complex then binds to both eNOS and nNOS resulting in release of NOS isoforms. In order for iNOS to be present in cells, they first need to be stimulated with an inducing agent, such as lipopolysaccharide (LPS). Transduction and transcription factors are activated resulting in the synthesis of new iNOS protein. iNOS protein has FAD, FMN, BH_4 and calcium tightly bound to the mature enzyme and therefore does not require additional cellular stimulation to produce NO. Excessive NO production results in accelerated metabolism to a family of potentially harmful reactive nitrogen species (RNS), including peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2), which both play very important roles in airway tissue injury.

Inflammatory diseases of the respiratory tract are commonly associated with elevated production of nitric oxide (NO) and increased indices of NO -dependent oxidative stress (85). Following the airway insult by MB, inflammation will occur and a range of inflammatory cells will be recruited to the inflammatory site. Macrophages, mast cells and epithelial cells release NO, and NOS are released by a number of inflammatory cells. The presence of inflammatory cells is also associated with increased oxidant injury. These phagocytes and other nonimmune and immune cells may release reactive oxygen and nitrogen intermediates that cause airway epithelial injury during inflammation (86).

1.7.4 Sodium metabisulphite effects on MMPs and TIMPs

As mentioned above, sodium metabisulphite (MB) can induce acute airway epithelial injury. When MB was applied to the frog palate surface, the insult of SO_2 caused airway inflammation (87). The lung system immune response results in the recruitment of a range of inflammatory cells (neutrophils, macrophages, eosinophils, lymphocytes) (88) to the inflammatory sites. Inflammatory cells release a range of mediators (MMPs, histamine, leukotrienes, nitric oxide, platelet activating factor (PAF), prostaglandins), chemokines (IL-8, regulated on activated, normal T expressed and secreted protein (RANTES), monocyte chemoattractant protein (MCP)), and cytokines (IL-1, tumour necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interferon (IFN)). Inflammatory mediators directly damage airway tissue (89), while chemokines and cytokines can act as the chemoattractants (90) for more inflammatory cells and activate these cells to release more inflammatory mediators. Airway epithelial cells can also be activated by cytokines and chemokines to synthesize and release MMPs (55). Once MMPs are released into the extracellular environment, they are activated, then break down the basement membrane and degrade the connective tissue in the airway epithelia (92). Thus, MMPs play a very important role in airway remodeling. An acute 10- to 160-fold increase of MMP-9 was detected in bronchial lavage fluid from patients with *status asthmatics* associated with free metallogelatinolytic activity (104). It also has been reported that increased activity of MMP-9 in alveolar macrophages occurs in experimental emphysema caused by surfactant protein D gene knockout (105).

As the tissue inhibitor of MMP-9 in most cells, TIMP-1 is secreted as a complex with MMP-9. The alteration of TIMP-1 level also happens in inflammatory diseases. Reports on TIMP-1 release during inflammation are diverse. Increased TIMP-1 expression has been reported in the sputum of patients with asthma and chronic bronchitis (106), while decreased TIMP-1 has also been demonstrated in bronchoalveolar lavage from the patients of nocturnal asthma (107). It is thus important to note that MMP/TIMP ratio in response to SO_2 induced inflammation may depend on the tissue and cell type.

1.8 EFFECTS OF CIGARETTE SMOKE ON AIRWAY INJURY

Cigarette smoking is the most important cause of COPD. Long-term exposure to cigarette smoke can cause airway injury and tissue damage (13, 94); however, the damage to the airway epithelia caused by acute cigarette smoke exposure is not very clear. This section will focus on a discussion of short-term cigarette smoke exposure injury to the airway.

1.8.1 Models of airway injury

Many studies have reported on the chronic effects of tobacco exposure (95, 96, 97, 98, 99), but the acute and early effects on cilia, mucus, and mucociliary clearance after active smoking or side-stream tobacco smoke exposure have not been studied. In general, the effects of ongoing tobacco exposure involve tissue inflammation, injury, healing and remodeling and may occur simultaneously. Human health consequences take multiple steps and several decades to be developed, so the changes occur slowly, basically free of symptoms. The exposed person does not feel the progressive effects, which take place at the molecular and ultra-structural levels and may not manifest noticeable symptoms for many years. However, as a consequence of continued exposure the cells and tissues, smokers may suffer progressive anatomical changes that could explain the functional changes occurring in the airways defence mechanisms. These interactive processes may also set the stage for subsequent development of malignant changes.

We are interested in developing an exposure model that will allow us to study the initial effects and mechanisms occurring in an epithelial tissue after exposure to environmental tobacco smoke. We also expect to have a better understanding of the mechanism by which ciliated epithelial cells are exfoliated after being exposed to tobacco smoke, as this may relate directly to impaired mucus clearance in several human airway diseases including chronic bronchitis and COPD. Because of the difficulties in studying mucociliary clearance in intact mammalian airways, once again the frog palate was used as the experimental model. As will be described in Chapter 3, fresh frog palate was put into the environment filled with cigarette smoke. Smoke was continuously absorbed into the environment to maintain the exposure. A scanning electron microscopy (SEM) study showed that ciliated cells started to slough from the epithelia with as little as one cigarette smoke exposure; however we were more interested in airway damage which is more acute and significant since it is much less studied compared as the chronic airway damage of CS, thus we used four cigarette exposures as our "application dose".

1.8.2 Mechanism of cigarette smoke induced injury

Cigarette smoke is a complex mixture of > 4,700 chemical compounds, of which free radicals and other oxidants are present in high concentrations (100). Free radicals are present in both the tar and the gas phases of cigarette smoke. The gas phase of cigarette smoke contains approximately 10^{15} radicals per puff, primarily of the alkyl and peroxyl types, and10^{20} oxidant molecules per puff in total (100). NO is one of oxidants that are present in cigarette smoke in concentrations of 500 to 1,000 ppm (100). NO reacts quickly the superoxide anion (O_2^-) to form peroxynitrite, and with peroxyl radicals to give alkyl peroxynitrites, both can contribute to tissue injury. The tar phase of cigarette contains more stable radicals, such as the semiquinone radical, which can react with oxygen to produce O_2^-, the hydroxyl radical, and hydrogen peroxide (100). The tar phase is also an effective metal chelator and can bind iron to produce the tar-semiquinone + tar-Fe^{2+}, which can generate hydrogen peroxide (102, 103). An oxidant/antioxidant imbalance has been suggested in the airway (101). The reaction of O_2^- and hydrogen peroxide (H_2O_2) in the presence of transition metal, usually ferrous iron (Fe^{++}), produces the hydroxyl radical ($^{\cdot}OH$). When catalyzed by neutrophil myeloperoxidase (MPO), H_2O_2 and a chloride form hypochlorous acid (HOCl). $^{\cdot}OH$ and HOCl are emphasized because both are extremely potent oxidants. HOCl is one of the most important oxidants associated with tissue damage. It is a very strong antibacterial acid but it also can affect the target site as airway epithelia and cause tissue damage and inflammatory response. If excessive HOCl is not removed, it can cause peroxidation of membrane lipids, depletion of nicotinamides, increased intracellular Ca^{2+} ions, cytoskeleton disruption, and DNA damage (13).

1.8.3 Cigarette smoke effects on MMPs and TIMPs

As mentioned above, cigarette smoke can induce an excessive oxidant burden to the respiratory system; and excessive HOCl can cause inflammation, and lead to tissue injury. This process may possibly involve MMPs and TIMPs. Investigators have demonstrated numerous effects of long term cigarette smoke exposure on the activation of MMPs and release of their inhibitors (TIMPs) in various cell types (108, 109, 143); however, the effect of acute cigarette smoke exposure on epithelial cells has not been well characterized. Increased MMP-9 level and decreased TIMP-1 level was found in alveolar macrophages of heavy smokers in comparison to non-smokers (108). In rat lungs, exposure to cigarette smoke induced increases in TIMP-1 mRNA levels (109). It also has been reported that when human vascular endothelial cells are exposed to cigarette smoke condensate, upregulation of MMP-9 gene occurs (110).

1.9 EPITHELIAL AND CILIAL DYSFUNCTION IN THE AIRWAYS

The respiratory system is a structurally complex arrangement of organs designed primarily for the intake of oxygen and the elimination of carbon dioxide. It can be anatomically divided into two main portions: the proximal conducting airway and the distal respiratory airway. The object of this section is to describe the proximal, conducting, non-gas-exchange portion of the respiratory system.

Changes in the ratio of MMPs/TIMPs and mucociliary clearance function of the epithelium may indicate a perturbation in the function of the epithelium itself. This section will focus on the role of conducting airway epithelium in normal physiology and in the pathophysiology of airway injury.

1.9.1 The airway epithelium in normal physiology

The airway epithelium lined with mucus is traditionally viewed as a barrier between the underlying tissues and the respiratory gases. Secretions of fluid and mucus, driven toward the mouth by cilia motion, serve to remove inhaled particles. In fact, the airway epithelium is a complex and active organ with metabolic, endocrine and structural functions. There are many species differences in the types, abundance and distribution of epithelial cells lining the conducting airways. The components of the apparatus in the central airways are, from top to bottom, the mucus layer, periciliary fluid layer, surface epithelial layer consisting of: (1) Ciliated cells, attached to the basal lamina and extending to the luminal surface, serve to generate mucous, and to move the overlying mucus cephalad; (2) Unciliated cells (basal cells and secretory granule-containing goblet cells), submucosal gland, and other specialized cells such as mast cells, neuroepithelial bodies (NEBs), Clara cells, etc. The whole epithelial apparatus plays an important role not only in barrier function but also in mucociliary clearance and mediator release. In normal airway epithelium, the cilia are about 5μm long, and their clawed tips beat in the periciliary fluid, contacting the underside of the mucus and moving it slowly (2-20mm/min) (111) toward the mouth, where it is swallowed or expectorated. Entrapped particulate matter moves with the mucus, and thereby, the airway is kept clean. The release of mediators is a recently discovered function of airway epithelium. Some of the mediators could have profound effects in neighbouring cell types (112).

6

1.9.2 Airway epithelia insults and ciliary dysfunction in airway injury

Numerous studies have documented the mechanisms of acute injury to the epithelial cells comprising the mucociliary escalator. To the healthy individuals, acute epithelial injury results from naturally acquired respiratory infections and intermittent exposures to irritants and pollutants in the ambient air. Host inflammatory responses to injury may result in morphologically evident changes that can be associated with transient functional decrements in mucociliary clearance and general respiratory health. Recurrent infections and/or continuing exposure to airborne gases and particulates can result in remodeling of the airway epithelial architecture in a way that may lead to persistent changes and severe adverse respiratory health effects and ultimately to death.

Ciliated cells are particularly vulnerable to injury. Studies have shown that ciliated epithelial cells obtained from patients with culture-documented viral upper respiratory infections exhibit transient changes in the microtubular organization of their airway cilia (113). Because optimal ciliary function is so closely attuned to ciliary organization, it follows that these defective cilia are limited in their capacity to clear the airways. Some experimental studies have shown that infecting bacterial pathogens attach to the respiratory mucosa and in some cases the attachment is preferential for ciliated cells (114). The mechanisms whereby bacteria cause epithelial injury have not been not fully characterized although it appears that the organisms or their metabolic products may able to suppress ciliary activity and/or to cause the loss of ciliated cells from the airway lining thus impairing mucociliary clearance. When airways are exposed to air pollutants, especially sulphur dioxide (SO_2), the membranes of individual cilia fuse with one another to form large dysfunctional compound cilia (115). Insults such as this eventually result in loss of entire ciliated cells from the epithelium and may lead, through a complex process, to chronic bronchitis, which may be characterized by mucus hyperplasia in which the ciliated cell population is replaced by secretory cells.

1.9.3 Evidence of epithelial insult and cilia dysfunction on the injured airway surface induced by polluted air and cigarette smoke.

There is a growing awareness in the scientific community about the relationships between air pollutant exposure and adverse respiratory health effects. While air pollution concerns seem most often to be associated with industrialized settings and automobile use, some common air pollutants such as sulphur and nitrogen dioxide derive from combustion of fossil fuels and may also be present in the home environment. **Figure 1.3** illustrates a form of ciliary injury called compounding that is typical of injury by gaseous pollutants, particularly sulphur dioxide. **Figure 1.4** showed the result of this insult, which is chronic bronchitis, characterized by mucus hyperplasia where the ciliated cell population is replaced.

Several investigators found that in children exposed to air pollutants, especially SO_2, ciliary abnormalities, including absent central microtubules, supernumerary central and peripheral tubules, ciliary microtubular discontinuities, and compound cilia can be demonstrated. A transudate was evident between epithelial cells, suggesting potential deficiencies in epithelial junction integrity (115). In a dog model, Heyder et al. (116) reported detectable lung and airway epithelial injury in SO_2-related environmental air pollutant exposure; ciliary dysfunction was also noted. Destruction of the protective ciliated airway epithelium in rat airway due to acute SO_2 poisoning has also been reported; epithelial injury, cilia beat frequency (CBF) decrease and cilia sloughing can be detected in the model (117).

Cigarette smoke induces excessive oxidants into the respiratory system, causes an oxidant-antioxidant imbalance, and finally leads to airway epithelial and lung parenchymal injury. In 1994, Sisson (118) et al. reported detectable cilia loss in bovine lung bronchoalveolar lavage (BAL) after *ex-vivo* acute cigarette smoke exposure. Investigators also reported that in a mouse model of cigarette smoke-induced COPD (119), loss of ciliated epithelial cells, infiltration of immune and inflammatory cells, such as macrophages, could be detected in the airways, which demonstrated the airway epithelial injury and ciliary abnormalities.

Collectively, the data imply that air pollutants, especially SO_2 and cigarette smoke, can induce airway insults and ciliary dysfunction in several tissue and experimental models.

1.10 EFFECTS OF OPHIOPOGONIS ON AIRWAY DISEASE AND INJURY

In previous studies in our lab, we employed an intervention in a quail model to simulate the pathogenic status of impaired mucociliary clearance with deterioration of mucus secretion. We evaluated the effect of Maimendong Tang (Ophiopogon Decoction, a herbal prescription therapy for COPD accompanied by viscous mucus secretion) on mucociliary clearance and mucus secretion in this model system (120). And since *Radix*

Ophiopogonis (OP) is regarded as the primary herbal ingredient of Maimendong Tang treatment, it is hypothesized that OP may be the most important mucoactive portion and manifest similar mucoactive effects (120,121).

Maimendong Tang, also named Ophiopogon Decoction, is a traditional Chinese herbal prescription therapy for COPD with mucociliary clearance impairment especially accompanied by viscous mucus secretion. Maimendong Tang is composed of *Radix Ophiopogonis, Rhizoma Pinelliae, Radix Ginseng, Radix Glycyrrhizae, Oryza Glutinosae* and *Fructus Ziziphi Jujubae*. OP is regarded as the primary herbal ingredient for Maimendong Tang to treat COPD in the clinic; it is hypothesized that *Radix Ophiopogonis* may be the most important mucoactive portion and manifest similar mucoactive effects to the whole prescription. This herb is the root tuber of Ophio-pogon japonicus (Thunb.) Ker-Gawl (family Liliaceae), and is grown in all parts of China. It is dug in summer, washed clean, stripped of the rootlets, dried in the sun, and used without further preparation. It has a sweet and slightly bitter flavour, acting on the lung, heart and stomach channels by moistening the lung, nourishing the stomach, promoting the production of body fluid, clearing away heat fire (a clinical term in Chinese medicine)to relieve vexation, and moistening the bowels to relieve constipation. It has been long used in the therapeutic treatment for COPD, vexation and insomnia, and diseases of digestive system (139).

1.10.1 Evidence of ophiopogonis benefit on mucociliary clearance in the injured airways

Due to its long-known effect on obstructive respiratory diseases as COPD, investigators in our lab have detected the effect of OP on airway mucociliary clearance and mucus secretion in anaesthetized quails (122). It has been found that either 10g/kg or 3g/kg of OP markedly attenuated the human neutrophil elastase (HNE)-induced decrease in mucociliary clearance time (MCT), and 10g/kg of OP significantly abolished the HNE-induced increases in fucose and protein contents of trachea lavage, which suggested that OP improves airway mucociliary clearance and that the improvement may, at least in part, be ascribed to the amelioration of airway mucus secretion. SEM study also showed that 10g/kg of OP can significantly decrease the area of cilia loss. In those studies, interestingly, OP manifested no obvious effect on intact airway epithelial cilia and airway mucus secretion in the physiological quail model but had an inhibitory effect on the damage to airway cilia and the development of airway mucus hypersecretion. These results suggest that the herb, as a natural product, has much less protective effect on normal airway epithelia compared with the substantial protective effect on abnormal airway epithelia.

1.11 REMODELING FACTORS IN AIRWAY INJURY

1.11.1 Ex-vivo studies

In the studies described in this thesis, we use excised frog palate to test mucociliary function. In this model, blood flow and circulation do not exist. The effect of circulation has been investigated primarily in a number of *ex vivo* studies that have compared tissue samples, taken before or after removal of the airway away from the body, on the function of epithelial mucociliary clearance. Evidence shows that frog palates maintained at the same temperature and humidity as *in vivo* for 20 minutes had the same mucociliary clearance function as a similar palate maintained in this condition *in vivo* for 24 hours (123). Indeed, many other investigators also investigate remodeling in *ex vivo* samples. Driss et al. (124) studied the remodeling of pulmonary artery in the rat *ex-vivo* model. Voisard et al. (125) also reported their ex-vivo study on the remodeling of pig heart.

1.11.2 Identity of remodeling factors

Airway remodeling is a critical aspect of wound repair in all organs, and represents a dynamic process that associates matrix production and degradation in reaction to an inflammatory insult (126) leading to remodeling either a normal reconstruction process (normal shape) or a pathological one (abnormal shape). For inflammatory respiratory disease, structural changes include extracellular matrix remodeling, epithelial desquamation, goblet cell hyperplasia, prominent smooth muscle, vascular remodeling and collagen deposition below the basement membrane. It is critical to understand the regulation of airway remodeling in airway injury, but its initiation and progression are not yet understood. Apoptosis is a process of cell death which functions to efficiently eliminate normal cells no longer required in remodeling tissues (127). The balance of cell recruitment and apoptosis during the healing response, and aberration of this process, appear to be important factors in lesion progression. MMPs are likely to be involved in the remodeling of injured airway. Other factors such as cytokines, cellular signalling pathway, growth factor, and inflammatory cells are also important to airway remodeling.

IL-11 is a pleiotropic cytokine that induces tissue remodeling with subepithelial fibrosis. It has been reported that IL-11 selectively inhibits antigen-induced eosinophilia, Th2-type inflammation, and vascular cell adhesion molecular-1 (VCAM-1) gene expression in pulmonary tissues (128). Signalling through G protein-coupled receptors (GPCRs) mediates numerous airway smooth muscle functions including contraction, growth, and "synthetic" functions that orchestrate airway inflammation and promote remodeling of airway architecture. Billington et al. recently examined the role of GPCR signalling and its regulation in asthma and suggested an integrative model, whereby an imbalance of GPCR-derived signals in ASM cells contributes to the asthmatic state (129). A number of proteoglycans and proteins present within the extracellular matrix of bronchial tissue are known to bind growth factors that are considered to contribute to the pathogenesis of airway inflammation and injury (130). The exact role of inflammatory cells such as eosinophils in airway remodeling is unclear. Typically in asthma, injury and inflammation are characterized by increased numbers of eosinophils in the airway, even under stable conditions, and eosinophils are recruited into the airway following allergen exposure. Eosinophils produce a variety of mediators that contribute to airway inflammation: leukotrienes, granule proteins, toxic oxygen products, and cytokines. In addition, metalloproteinases, collagenases, and growth factors from eosinophils may be important in regulating matrix tissues (131). Investigators have reported that mice that were depleted of circulating neutrophils had markedly less lung inflammation and demonstrated little evidence of LPS-induced airway hyperreactivity or airway remodeling (expansion of the subepithelial matrix); in contrast, neutrophil-replete mice developed airway inflammation, hyperreactivity, and remodeling after a prolonged exposure to LPS. These findings indicate that neutrophils can also contribute to airway remodeling and hyperreactivity (144). Investigators also have demonstrated that anti-IL-5 treatment, which can partially inhibit eosinophil recruitment, reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics (132).

1.11.3 The role of MMPs in remodeling

Connective tissue cells produce and secrete an array of macromolecules forming the extracellular matrix (ECM) (133), a complex network filling the extracellular space of the submucosa. The macromolecules that constitute the ECM consist of fibrous proteins (collagen, elastin) and structural or adhesive proteins (fibronectin and laminin) embedded in hyaluronic acid. The ECM is a dynamic structure, and equilibrium between synthesis (134) and degradation of ECM components is required for the maintenance of its homeostasis. MMPs are major proteolytic enzymes that are involved in ECM turnover, due to their ability to cleave all the proteins constituting ECM (135). MMP-9 is the major MMP found in the airways and its major inhibitor is TIMP-1. MMP-9 is expressed at high levels by several types of inflammatory cells (neutrophils, lymphocytes, eosinophils, mast cells and macrophages) and epithelial cells. It may locally degrade the subendothelial basement membrane during the processes of inflammatory cell extravasation from the vascular compartment into tissues. It has been found that migration of resting T cells across the basement membrane in vitro is inhibited by an MMP inhibitor (136). This is likely due to inhibition of MMP-9 because it is the major type IV collagenase expressed by resting T cells. The turnover of ECM is regulated, at least in part, by MMP-9 and TIMP-1. Vignola et al. (137) have reported that in sputum of asthmatic and chronic bronchitis patients the level of TIMP-1 is significantly increased compared with that of control subjects, and that the molar ratio between MMP-9 and TIMP-1 is significantly lower than in control subjects, suggesting the existence of a protease-antiprotease imbalance in these diseases. However, investigators also have reported that the macrophages from COPD patients tend to release more MMP-9 but less TIMP-1 than healthy people (108). In asthma, investigators found an inverse correlation between the MMP-9/ TIMP-1 molar ratio and airway obstruction (138). It is important to note that even with the same MMP-9/ TIMP-1 molar ratio, the activation of MMP-9 and release of TIMP-1 will be different in different cell types and different species.

The role of MMPs in mediating acute airway injury and remodeling is likely to be significant. It also has been demonstrated that the MT1-MMP/MMP-2/TIMP-2 system plays a significant role in the MMP-mediated extracellular matrix degradation and tissue remodeling of emphysematous lungs, and thus may contribute to the weakening of lung parenchyma and lead to the formation of emphysema (146). Investigators also reported that IL-13–induced airway remodeling such as alveolar enlargement, lung enlargement, and compliance alterations were markedly decreased in the MMP-9 and MMP-12 (elastase) knockout mice (147). Furthermore, airway insult factors such high concentration of SO_2 and cigarette smoke may alter the epithelium reactivity and stimulate the activation of MMPs from epithelium. This intriguing possibility provides a new perspective on the role of MMPs in the pathophysiology of airway injury.

1.12 SUMMARY

Sodium metabisulphite and cigarette smoke can induce airway injury. The injury of sodium metabisulphite involves inflammation, while the injury of cigarette smoke is associated with oxidant stress. MMPs have a diverse range of biological functions, and are important to ECM remodeling. A shift in the balance between MMPs and their inhibitors may have serious implications on airway epithelial remodeling, and may contribute to the pathophysiology of airway injury.

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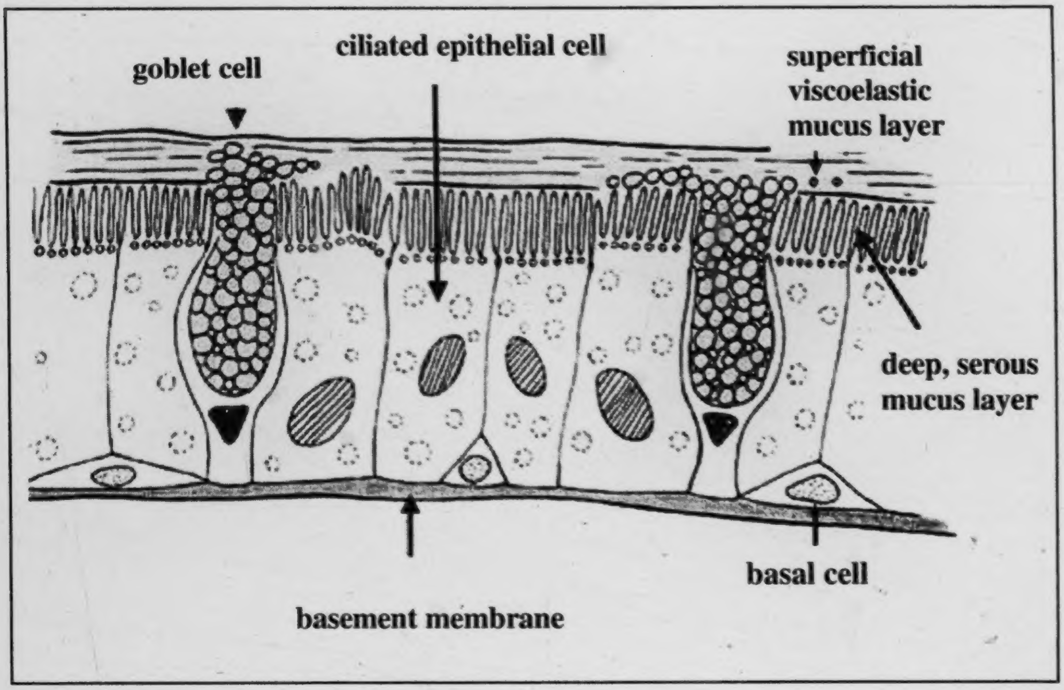

Figure 1.1 The structure of frog palate epithelium.

The diagram illustrates ciliated cells (attached to the basal lamina and extending to the luminal surface, serve to generate mucous, and to move the overlying mucus cephalad.), unciliated cells (basal cells and secretory granule-containing goblet cells), basement membrane, mucus layer and periciliary serous layer. The mucus was secreted by goblet cells, and transported by the cilia beating.

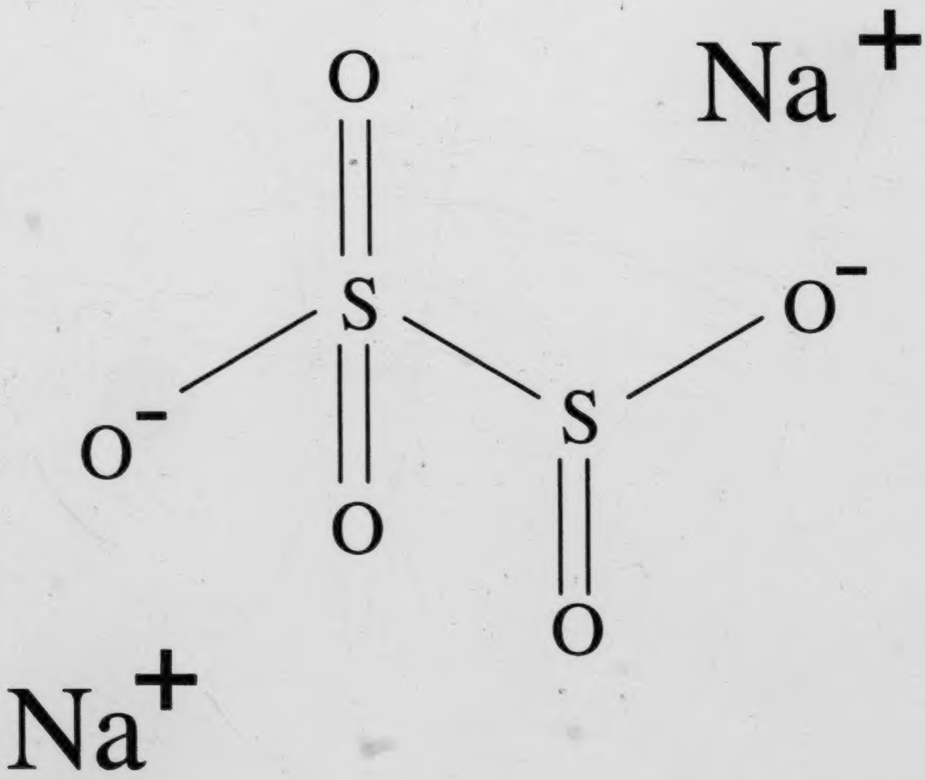

Figure 1.2 Sodium metabisulphite ($Na_2S_2O_5$)

Melting Point: 150°C
Specific Gravity (H_2O=1): 1.4
Solubility: 45% in water at 20 °C
Decomposition Temperature: Approx 150 °C
pH: 4.5 (1% solution).

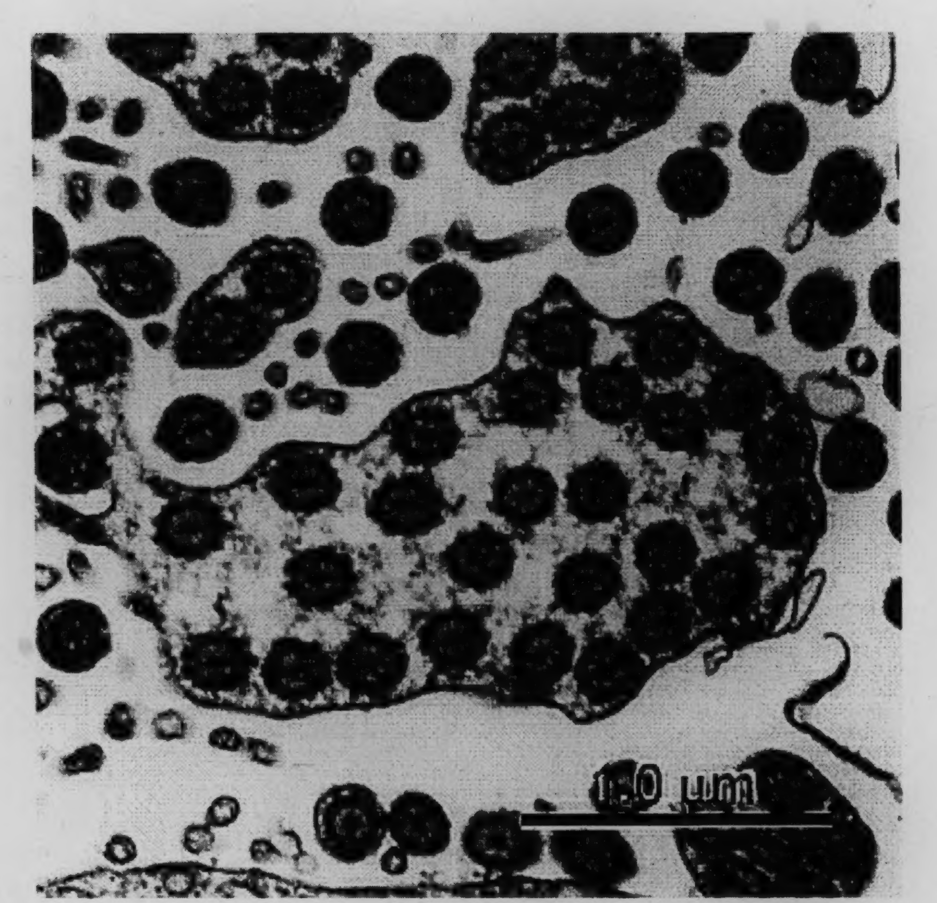

Figure 1.3 Cilia injury: compounding

The panel above illustrates a form of ciliary injury called compounding that is typical of injury by gaseous pollutants, particularly sulphur dioxide, a by-product of fossil fuel combustion. Here, the membranes of individual cilia have fused with one another to form large dysfunctional compound cilia. Insults such as this eventually result in loss of the entire ciliated cell from the epithelium and may lead to chronic bronchitis.

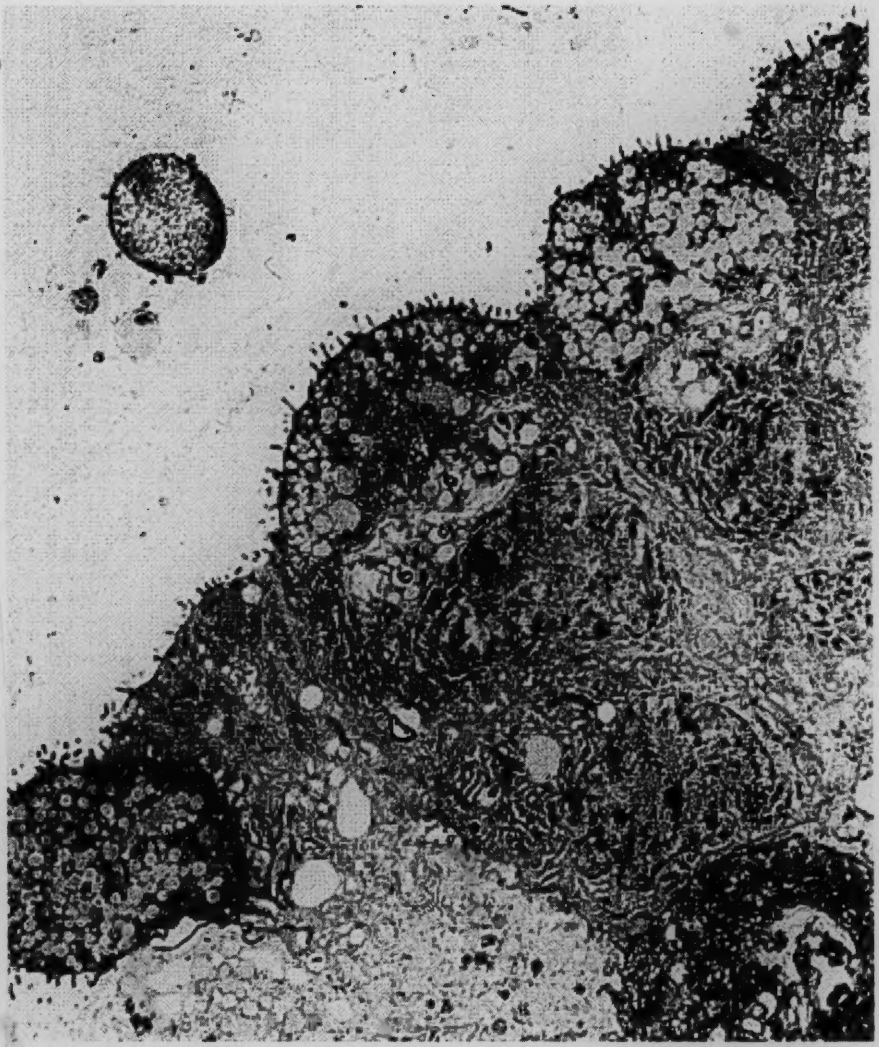

Figure 1.4 Airway epithelial injury in chronic bronchitis.

Chronic bronchitis characterized by mucus hyperplasia in which the ciliated cell population is replaced by secretory cells.

The MMP Family

Common name	***Other name***
Collagenases	
Collagenase-1	MMP-1
Collagenase-2	MMP-8
Collagenase-3	MMP-13
Collagenase-4	MMP-18
Gelatinases	
Gelatinase-A	MMP-2
Gelatinase-B	MMP-9
Stromelysins	
Stromelysin-1	MMP-3
Stromelysin-2	MMP-10
Membrane-type MMPs	
MT1-MMP	MMP-14
MT2-MMP	MMP15
MT3-MMP	MMp-16
MT4-MMP	MMP-17
Other MMPs	
Matrilysin	MMP-7
Stromelysin-3	MMP-11
Metalloelastase	MMP-12
Enamelysin	MMP-20
MMP-19	
MMP-21	
MMP-22	

Table 1.1 The MMP family.

PSI vs. Air Pollutants

PSI value	Effect on health	24-hr.PM $_{10}$	24-hr.SO_2	8-hr.CO	1-hr.O_3	1-hr.NO_2
		($\mu g/m^3$)	(ppm)	(ppm)	(ppm)	(ppm)
50	Good	50	0.03	4.5	0.06	-
100	Moderate	150	0.14	9	0.12	-
200	Unhealthful	350	0.3	15	0.2	0.6
300	Very-Unhealthful	420	0.6	30	0.4	1.2
400	Hazardous	500	0.8	40	0.5	1.6
500	Hazardous	600	1	50	0.6	2

Table 1.2 Pollution standard index by World Health Organization (WHO)

CHAPTER 2. THE EFFECTS OF SODIUM METABISULPHITE INDUCED INJURY AND OPHIOPOGONIS INCUBATION ON MUCOCILIARY CLEARANCE FUNCTION.

2.1 INTRODUCTION

Particle clearance in the airways is dependent on mucus and cilia (2). Various factors such as cilia beat frequency, mucus secretion rate and mucus properties must be coordinated to result in normal function and effective mucociliary clearance (3). Mucociliary clearance time (MCT) is one of the most commonly used measurements of mucus clearance function. It is the time recorded for the displacement of moving particles over an airway segment, and from it mucociliary clearance velocity (MCV) can be determined (2). Because of the difficulties in studying mucociliary clearance in intact mammalian airways, investigators long ago turned to more easily accessible system such as frog palate in which to study the principles that regulate mucociliary transport. The frog palate was best choice because of its ready accessibility and its many similarities with higher systems. Stewart (4) published the first description of the weight-carrying capacity of frog palate cilia in 1948. Since then, a great number of studies have been carried out (1, 2, 3, 5, 6, 7) that provide the basis for the use of the excised, mucus-depleted frog palate as a practical model for testing the inherent clearability of mucus collected from various sources. In this study we report on the development on a novel frog palate model, which has application for the investigation of basic mechanisms related to mucociliary transport and for the testing of mucoactive or protective agents, in checking herbal drugs.

As described in Chapter 1, air pollution contributes to a range of lung diseases. Major health concerns associated with exposure to high concentrations of sulphur dioxide (SO_2), one of the most important components of air pollution, include effects on breathing, respiratory illness, alterations in pulmonary defences, etc. (8). Chronic inspiration of or long-term exposure SO_2 induces airway inflammation and epithelial injury (9, 10). However, the effect of acute, high-concentration SO_2 exposure on normal airway physiology and mucociliary function, which can occur in the occupational situations, is still not clear. Sodium metabisulphite (MB), dissolved in water, releases SO_2 *in situ*, producing similar lesions to classic SO_2 gas induced bronchitis (3). MB has been employed in other airway injury models such as guinea pig (11) and the rat (12) to study hypersecretion and hyperplasia, which affects morbidity and mortality in patients suffering from asthma, chronic bronchitis and cystic fibrosis. To create an injury model from the fresh frog palate model, we administrated a topical solution of sodium metabisulphite to the palate.

It has been suggested that herbal drugs generally show minor side effects, compared to synthetic or purified pharmaceutical agents and are therefore particularly suited to treatment of chronic disorders (13). While many traditional Chinese herbal drugs have a long history of clinical effectiveness, scientific evidence to justify their use is lacking. Furthermore, it is difficult to demonstrate their specific effects under normal conditions, as their traditional beneficial effects have been shown in pathologic states. Therefore an injury model may be particularly suited for the study of herbal drugs, in addition to other agents.

Tai et al. (13) investigated the effects of a decoction, called Maimendong Tang, which consists of six herbs, in a quail model of mucociliary transport. This blended medicine is composed of *Radix Ophipogonis, Rhizoma Pinelliae, Radix Ginseng, Radix Glycyrrhizae, Oryza Glutinosae* and *Fructus Ziziphi Jujubae* (14). This agent has been shown to have beneficial effects on airway clearance and has been traditionally clinically employed in the treatment of chronic obstructive pulmonary diseases associated with the development of viscous sputum. *Radix Ophipogonis* (OP) is regarded as the primary herbal ingredient for Maimendong Tang to treat COPD in the clinic. It is hypothesized that OP may be the most important mucoactive and protective portion and would thus manifest similar mucoactive and protective effects to the blended prescription. In this study we describe the development of the frog palate injury model, with particular emphasis on the effect of the injury on mucociliary transport. We also present the results of our investigation of OP on this injury, and show that OP may have beneficial effects on mucociliary transport through an inhibitory effect on disrupted airway cilia structure and function.

2.2 MATERIALS AND METHODS

2.2.1 Animal model preparation

Bullfrogs (*Rana catesbiana*) were sacrificed using the double pithing method (15). Immediately after sacrificing the frog, the palate was excised by disarticulation of the jaw and removal of the upper part of the head by cutting with scissors through from the junction of the posterior pharynx and esophagus out to the skin of the back. The excised palate was then placed either into the frog Ringer (FR) or the Ophiopogonis (OP) decoction. FR solution was 2/3 Ringers + 1/3 distilled water, with an osmolarity of 206.5mOsm/L, containing 98.3 mM of NaCl, 2.7mM of KCl, and 1.5mM of $CaCl_2$. For the OP incubation, the solution was 2/3 Ringers +1/3 OP decoction (1mg/ml). OP, which was imported from China by Bao Shing Chinese Herbal Ltd. (Edmonton, AB, Canada), was extracted by twice boiling in water.

Positioning the palate vertically in a small incubation dish, which allowed the solution to contact the base of the palate, but not the ciliated palate surface, carried out incubation of the palate. Three millilitres of solution were sufficient to immerse the base of the palate. The dish was then placed in a 50 ml glass beaker, covered with parafilm and placed in a fridge at 4°C overnight. When the palate was taken out the next day, it was placed in a petri dish on a piece of FR soaked gauze.

2.2.2 Experimental protocol (drugs)

A. Control model

The palate incubated in FR or OP was taken out incubation and stabilized in the observation chamber for 15 minutes. It was then topically applied with an amount of FR, usually 2μl to 5μl depending on the size of the palate (n=3). Solutions applied to the palate were proportional to the area of the palate. In the present experiments, FR was used as a control solution. Normally, we left the palate for 2 minutes before measuring the MCT.

B. MB injury model

The palate incubated in FR or OP was taken out and stabilized in the observation chamber for 15 minutes, and was topically applied a certain amount of FR as described above (n=3). MCT was measured to make sure that it was in the normal range. Sodium metabisulphite (MB) was made up in FR to arrive at a stock solution with a concentration of (10^{-1}M or 10^{-2}M). The same amount of MB (10^{-2}M or 10^{-1}M) solution as FR was topically applied to the palate and MCT was recorded.

2.2.3 Mucociliary clearance observation

Mucociliary clearance observation chamber

In order to achieve our goals, we chose an *ex-vivo* preparation of frog palate (*Rana catesbiana*). After the frog palate was excised, it was placed in an acrylic chamber (see **Figure 2.1**, 20cm height, 30cm width, 20cm depth) maintained at a constant temperature (22°C to 24°C) and continuously humidified with FR, maintained with an aneroid hygrometer (Bacharach 15238, Pittsburgh, PA). A microscope was placed above the chamber upper surface in order to observe the mucociliary clearance movement; a scale was placed in the microscope to measure the displacement of the mucus. (1)

MCT and MTV measurements

Normal mucociliary clearance time (MCT) was measured by applying a droplet of FR to the frog palate. FR was used here as a control solution compared with other agents used later. The volume of the FR applied was proportional to the total epithelial area of this palate (estimated by measuring across the lateral-most borders of the jaw at the base of the palate and calculating the area of the equivalent half-circle). Usually the area of the palate varied from 3.5-6 cm^2, which corresponds to the volume of the solution applied from 2µl to 5µl. Normally, after a solution was applied to the palate, two minutes was allowed to elapse for the solution to be distributed over the palate before measurements of mucus transport were begun. This distribution was achieved, not by normal diffusion, but by active movement of the ciliated epithelium, which covered the entire surface of the palate. Application of 1.5 μl of bromphenol blue in FR showed that two minutes was sufficient for the solution to be carried from the top of the palate to base by ciliary action. When two minutes had elapsed, a drop of clear mucus (diameter 1-2mm) was collected from the cut edge of the palate and was placed on the top of the palate near the mouth side.

In order to visualize the movement of the mucus on the palate, carbon particles were applied on top of the mucus by a needle. A stereoscopic microscope was used to investigate the transport of the mucus drop from the top to the base of the palate (mouth side to cut side). Once the mucus transport reached a steady rate, MCT was determined by recording the time for the displacement of carbon particles by 5mm. At least 5 MCT measurements were recorded to arrive at a mean MCT after each treatment. The increase of MCT compared with the control time suggests a slowing down of the mucociliary clearance rate. MTV (mucus transport velocity) was calculated by dividing the distance the mucus travelled (5mm) by MCT.

2.2.4 Statistics

One way ANOVA was used to compare among three groups. Student's t-test was used to compare between two groups. Significance was considered when $p<0.05$.

2.3 RESULTS

The MCTs of each group are summarized in **Table 2.1**.

2.3.1 Comparisons of normal and sodium metabisulphite injured palate for mucociliary clearance function under different pre-incubations

For the FR-incubated samples, significant difference was confirmed by one way ANOVA among the MCT with the application of FR, MB (10^{-2}M) and MB (10^{-1}M) ($p<0.001$, $n=3$, **Figure 2.2**). A minimal effect on MCT ($p=0.069$, $n=3$) compared to control (FR application) was detected after application of MB (10^{-2}M). MCT was significantly increased (~150%, $p<0.001$, $n=3$) after MB (10^{-1}M) application compared to control.

For the OP-incubated samples, significant difference was confirmed by one way ANOVA among the MCT with the application of FR, MB (10^{-2}M) and MB (10^{-1}M) ($p<0.001$, $n=3$, **Figure 2.3**) No significant difference of MCT was detected between the control samples and MB (10^{-2}M) treated samples ($p=0.365$, $n=3$). MCT was significantly increased after application of MB (10^{-1}M) (~80%, $p<0.001$, $n=3$) compared to the control.

2.3.2 The effect of ophiopogonis incubation on mucociliary clearance function for normal and sodium metabisulphite injured palate

We compared MCT of the FR-incubated control group and the OP (1g/ml)-incubated control group (**Figure 2.4A**). MCT of the FR-incubated MB (10^{-2}M) treatment group and the corresponding OP (1g/ml)-incubated MB (10^{-2}M) treatment group was also compared (**Figure 2.4B**). Finally, MCT of the FR-incubated MB (10^{-1}M) treatment group and the OP (1g/ml)-incubated MB (10^{-1}M) treatment group was compared (**Figure 2.4C**).

The FR-incubated and OP (1g/ml)-incubated control group showed no significant difference (p=0.78, n=3) in MCT (**Figure 2.4A**). There was no significant effect on MCT (p=0.455, n=3) of varying incubation in the MB (10^{-2}M) treatment groups (**Figure 2.4B**). MCT was significantly reduced (~35%, p<0.001, n=3) in the OP (1g/ml)-incubated MB (10^{-1}M) treatment group compared with the FR-incubated group given the same treatment (**Figure 2.4C**).

2.4 DISCUSSION

In our studies, frog palates incubated in FR and treated by MB (10^{-1}M) demonstrated significantly increased MCT compared to FR-incubated control. This indicates that sodium metabisulphite can perturb normal mucociliary activity on the palate. Furthermore, the increase of MCT was recorded between two and ten minutes after application, which indicated that the injury to the frog palate epithelium induced by MB was very acute. We detected a minimal increase of MCT in the FR-incubated MB (10^{-2}M) treatment group compared with the control group; however, this difference was not significant. This suggests that in order to induce the acute, high concentration SO_2 injury to the frog palate epithelium, MB (10^{-1}M) was the more appropriate dose to use.

A second part of the model development was to derive a method to deliver a "test drug" to the palate tissue without actually directly applying the drug to the palate surface. The reason for this was to be able to distinguish between the systemic action of the herb and the topical effects of MB or FR on mucus transport. The injury model was particularly suited to investigation of traditional herbal drugs, which manifest little effect under normal conditions but are clinically effective in pathogenic states (13, 14). The herb, OP, which had been used traditionally in the treatment of COPD, was investigated for its action on mucociliary transport in the frog palate injury model and was shown to have no effect on MCT of the control groups and the MB (10^{-2}M) treated groups. However, for the group treated with MB (10^{-1}M), the MCT was dramatically decreased in the OP (1g/ml)-incubated group compared with the FR-incubated group. This result suggested that OP had a beneficial effect on the MB-treated palate. This protective/restorative effect of OP could be due to a mucoactive effect or to protection of the cilia bed. Although incubated in OP, the tissues treated with MB (10^{-1}M) were still found to have significantly increased MCT compared the OP (1g/ml)-incubated controls. This suggested that the OP could only partially block the injurious effect induced by MB (10^{-1}M).

The acute effect on mucociliary clearance function occurred rapidly after the application of MB to the palate, where MCT was seen to increase dramatically in the FR or OP pre-incubated groups. The cause of the acute injury is unclear, although pH may be a factor. MB (10^{-1}M) with a pH 4.5, when it was applied to the palate, we detected a reduced pH of the palate surface by 0.5 units to 6.4. The time frame and the magnitude of the pH change may have been adequate to influence ion channels and stimulate the release or up-regulation of inflammatory mediators, as has been previously reported (12). In an *in vitro* system, it was demonstrated (16) that the ciliary beat frequency of bronchial cells was stable between pH 7.5 and 10.5. At lower pH values, ciliary beat frequency decreases significantly. The mechanism of the increase in MCT may also involve the release of oxidants (such as nitric oxide) by epithelial cells (17, 18, 21) or from typical inflammatory cells (19). It is also suggested that the products of H_2O_2 may cause activation of precursor forms of collagenase or gelatinase, leading to breakdown of the extracellular matrix. Thus MB may activate or mediate the activation of matrix metalloproteinases (MMPs) resulting in extracellular matrix degradation. MMPs have been implicated as an important mediator of acute lung injury. Foda et al. (20) showed that mechanical stress resulted in the expression and release of gelatinases from epithelial and endothelial cells in the rat lung. The release of MMPs is part of an inflammatory response, preceding remodeling in the lung. In the present study, MMPs activity may be increased by inflammatory mediators and they may play a role in the loss of ciliated epithelium. Further studies to investigate the effects of MMPs on mucociliary clearance will be described in Chapter 3.

In conclusion, we have reported on a novel frog palate injury model which may be particularly suited for studies of herbal drugs, the actions of which may be more clearly discerned under pathological or injury conditions. We measured the MCT from control and MB (10^{-2}M) and MB (10^{-1}M) treated groups pre-incubated in FR or OP. We demonstrated increase of MCT in the MB (10^{-1}M) treated groups compared to controls whether they had been incubated in OP or not. The increase in MCT occurred shortly after the application of MB, suggesting that MB (10^{-1}M) can induce an acute epithelial injury, which is similar to the damage of SO_2 that can occur in airway models of occupational SO_2 exposure. We also observed that MB (10^{-1}M) treated samples showed reduced MCT in the OP (1g/ml)-incubated samples compared to the FR-incubated samples, indicating at least a partial blockade of the deleterious effects of SO_2 release. Finally we detected that OP-incubation has little or no effect on the mucociliary clearance function in the control groups and the MB (10^{-2}M) treated groups, reflected in our study by no significant changes of MCT. We suggest that the herb, *Radix Ophiopogonis,* as a natural product, has much less protective effect on normal epithelium or slightly injured epithelium compared with the substantial effect on abnormal epithelium.

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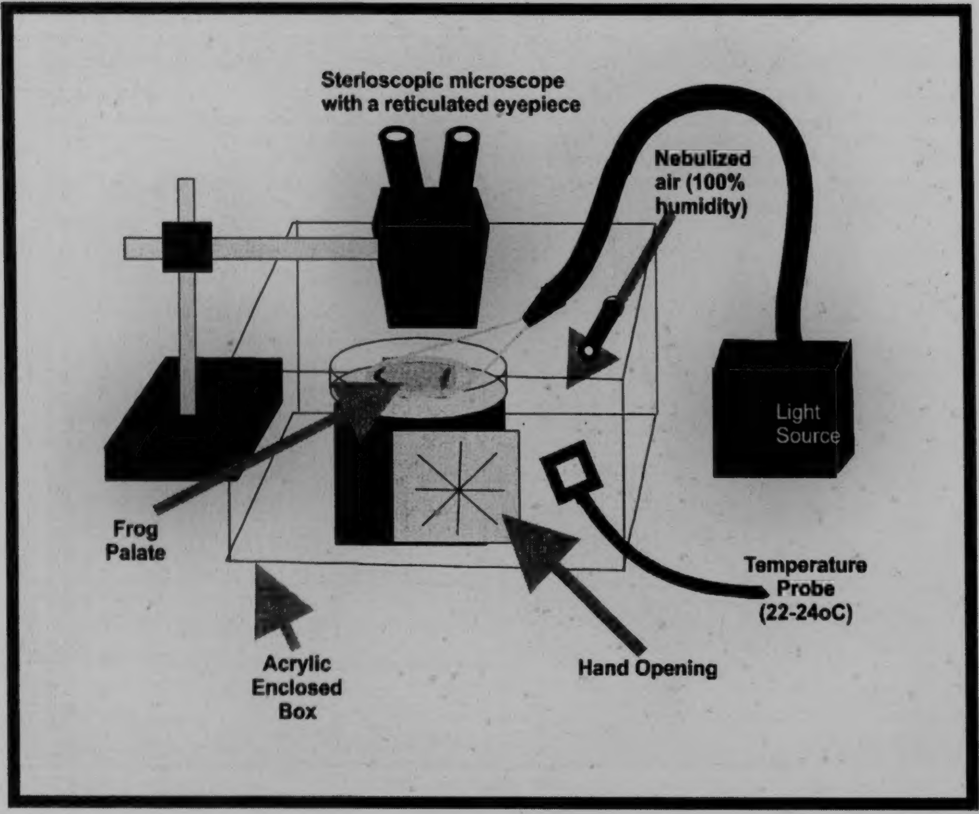

Sterioscopic microscope with a reticulated eyepiece
Nebulized air (100% humidity)
Light Source
Frog Palate
Temperature Probe (22-24oC)
Acrylic Enclosed Box
Hand Opening

Figure 2.1 Mucociliary` observation chamber

This diagram shows an acrylic chamber (20cm height, 30cm width, 20cm depth) maintained at a constant temperature (22°C to 24°C) and continuously humidified with nebulized FR, maintained with an aneroid hygrometer (Bacharach 15238, Pittsburgh, PA). The excised frog palate was placed in the center of the chamber. A microscope was placed above the chamber upper surface in order to observe the mucociliary clearance movement; a scale was placed in the microscope to measure the displacement of the mucus.

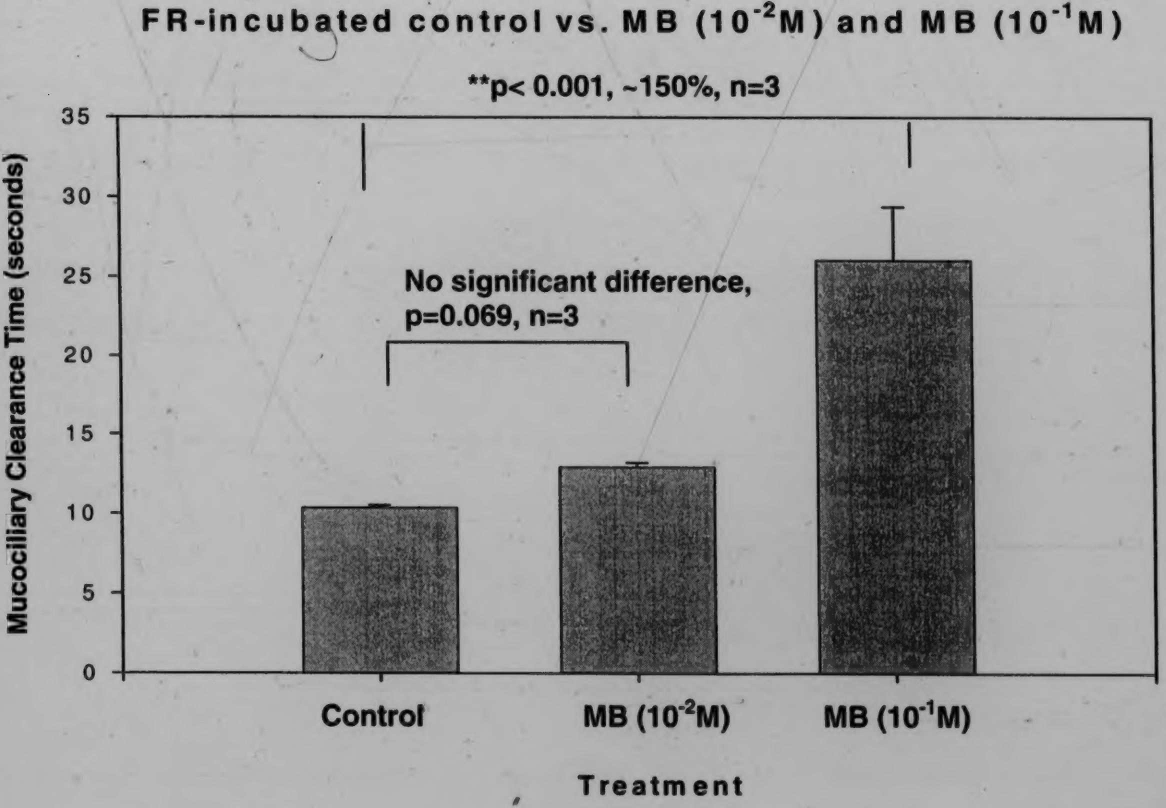


Figure 2.2 Comparison of mucociliary clearance Time, FR-incubated control vs. MB (10^{-2}M) vs. MB (10^{-1}M)

Mucociliary clearance time (MCT) from frog palates of FR pre-incubated (overnight) control, MB (10^{-2}M) treatment and MB (10^{-1}M) treatment (n=3, control; n=3, MB (10^{-2}M); n=3, MB (10^{-1}M)). The data are presented as means ±SEM of the MCT. **p< 0.001 at MB (10^{-1}M) treatment for MCT.

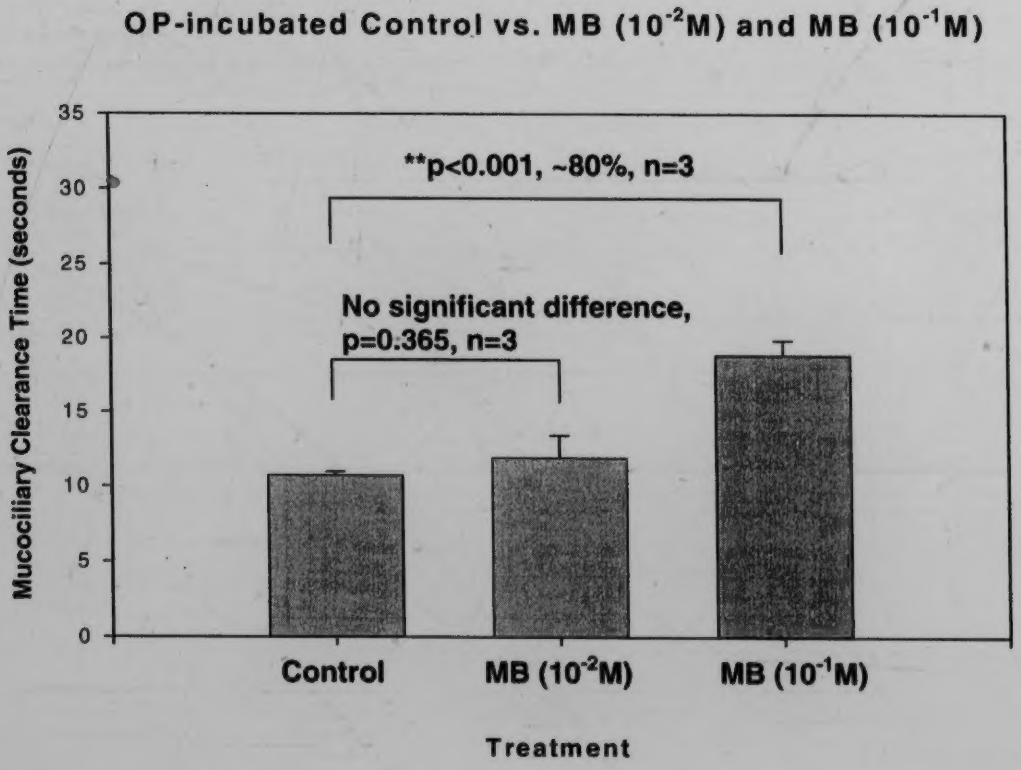
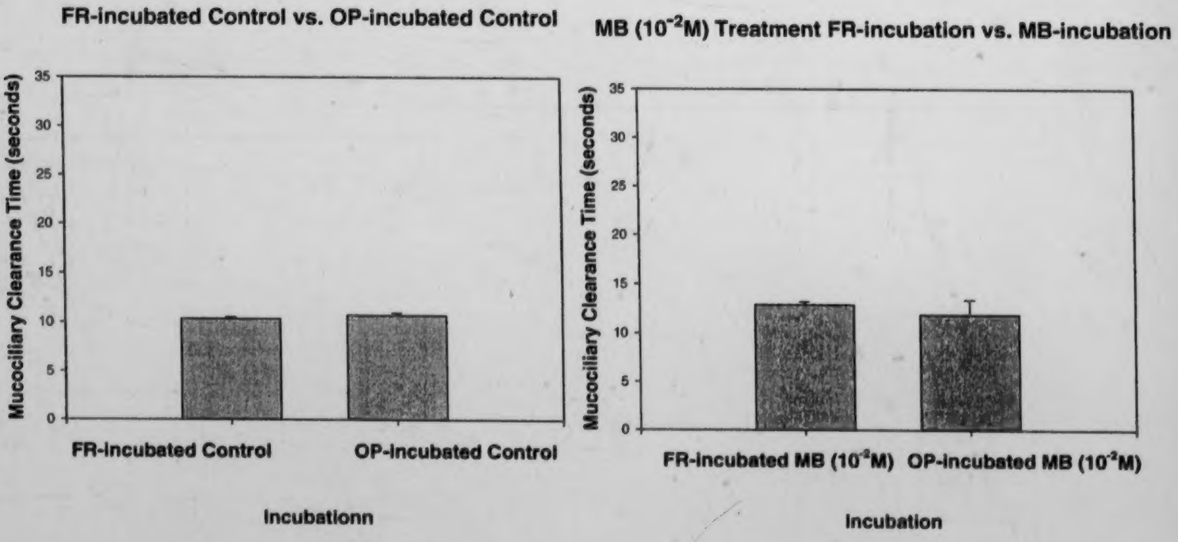


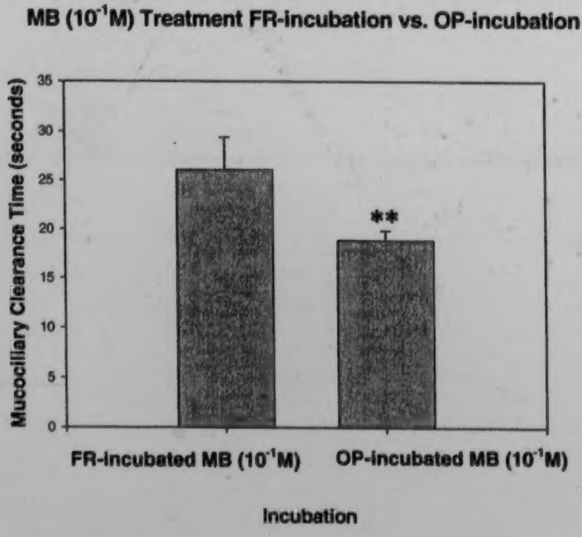
Figure 2.3 Comparison of mucociliary clearance time, FR-incubated control vs. MB (10^{-2}M) vs. MB (10^{-1}M)

Mucociliary clearance time (MCT) from frog palates of OP pre-incubated (overnight) control, MB (10^{-2}M) treatment and MB (10^{-1}M) treatment (n=3, control; n=3, MB (10^{-2}M); n=3, MB (10^{-1}M)). The data are presented as means ±SEM of the MCT. **$p < 0.001$ at the MB (10^{-1}M) treatment.


FR-incubated Control vs. OP-incubated Control

MB (10^{-2}M) Treatment FR-incubation vs. MB-incubation

(A) No significant difference, p=0.78, n=3 **(B) No significant difference, p=0.455, n=3**


MB (10^{-1}M) Treatment FR-incubation vs. OP-incubation

(C) **p< 0.001, ~35%, n=3

Figure 2.4 The effect of OP-incubation on mucociliary clearance function for normal and MB injured tissue.

Mucociliary clearance time (MCT) of the frog palates control pre-incubated (overnight) in FR or OP (n=3, FR; n=3, OP; **A**). Also shown is MCT of the frog palates treated by MB (10^{-2}M), pre-incubated (overnight) in FR or OP (n=3, FR; n=3, OP; **B**). MCT of the frog palates treated by MB (10^{-1}M) pre-incubated in FR or OP (n=3, FR; n=3, OP; **C**). The data are presented as means ±SEM of the MCT. ** $p<0.001$ for MB (10^{-1}M) treatment.

Mucociliary Clearance Time (MCT, seconds)

Application	FR-incubated (n=3)	OP-incubated (n=3)
FR-treated	10.36±0.15	10.72±0.24
MB (10^{-2}M)-treated	12.9±0.29	11.92±1.48
MB (10^{-1}M)-treated	26.04±3.34**	18.87±1.00**

Table 2.1 Mucociliary clearance time of each group in the experiment

FR= frog ringer solution; MB= sodium metabisulphite solution; *$p < 0.001$ vs. FR-incubated, FR-treated control.

CHAPTER 3. THE EFFECTS OF SODIUM METABISULPHITE-INDUCED INJURY AND OPHIOPOGONIS INCUBATION ON EPITHELIAL RELEASE OF MATRIX METALLOPROTEINASES AND THEIR TISSUE INHIBITORS

3.1 INTRODUCION

As demonstrated in the previous chapter, sodium metabisulphite can induce acute injury to epithelium, as evidenced by a rapid increase in MCT. Furthermore, OP had beneficial effects on preserving MCT in the MB (10^{-1}M) model. In this chapter we will explore whether this process might involve matrix metalloproteinases (MMPs).

MMPs are released by a variety of cells including epithelial cells (1), and require cleavage or reconfiguration of the N-terminal propeptide for full activation (2, 3). Their activity is regulated at multiple levels including transcription, secretion, activation and inhibition by specialized proteins called tissue inhibitors of metalloproteinases (TIMPs), which bind to the MMP active site (2). The resulting steric hindrance prevents interaction of the MMP with components of the extracellular matrix (ECM) (4). The gelatinases, MMP-2 and MMP-9 are extensively involved in ECM remodeling through degradation of basement membrane collagens (5) and the balance between MMPs and their inhibitors is likely to determine the extent of this remodeling within the airway epithelium (6).

With an overall hypothesis that dysregulation of matrix remodeling contributes to epthelial injury, we specifically hypothesize that in the epithelial tissue incubated with FR and injured by MB, the activation of MMPs will be increased and/or there are decreased level of TIMPs compared to normal tissue. We further hypothesize that in the epithelial tissue incubated with OP and injured by MB, the activation of MMPs is also increased compared to OP (1mg/ml)-incubated control, but much less than the FR-incubated samples with the same treatment; and/or there are decreased levels of TIMPs compared to the control but more than in FR-incubated samples. There are few researches has been done investigating the results of the application of MB on mammalian tissues, however, due to the similarity of the structures between frog palate and human conductive airways, the same result as the frog might be anticipated when MB is applied to mammalian tissues. Because of the potential effect of dose-dependent differences between normal and MB-injured epithelium, we tested our hypothesis at two concentrations of MB, 10^{-2}M and 10^{-1}M.

3.2 MATERIALS AND METHODS

3.2.1 Tissue collection and experimental protocol

Mucociliary clearance experiments were carried out as described in last chapter. After the MB or FR was applied, the epithelial tissue was carefully separated from the palate musculature, and two pieces of tissue (about 5mm × 15mm) were sectioned from the central part of the palate (as close to the application site as possible), frozen in liquid nitrogen immediately and stored at –80°C for zymography and Western blot studies.

3.2.2 Scanning electron microscope (SEM) studies

A further small piece of frog palate epithelial tissue (about 2mm × 8mm) was also taken from the palate, as close to the application site as possible. This latter sample was placed in 2.5% glutaraldehyde solution immediately after collection and stored in a refrigerator at 4°C until processing. Briefly, the sample was post-fixed in 1% osmium tetroixde in Millonig's buffer at room temperature for one hour. It was then washed briefly in distilled water and dehydrated in a series of ethanol (50-100%), ten minutes at each concentration, followed by two additional periods of absolute ethanol (10 minutes each). The sample was further dehydrated by critical point drying at 31°C for 5-10 minutes, then mounted on a specimen holder for SEM and dried overnight in a vacuum desiccator. In the final stage of preparation for viewing, the sample was sputter coated with gold (Edwards model S150B Sputter Coater). It was examined with a Hitachi S-2500 scanning electron microscope. Digital images were acquired and saved to a computer for further study.

3.2.3 Gelatin zymography

Tissue samples were collected as described above and protein concentrations were measured. The MMP-2 and MMP-9 levels in the epithelial tissues were evaluated by zymography according to established methods (7, 8). Zymography detects both the proenzyme and mature forms of MMPs, distinguished on the basis of molecular weight (9) and it quantitatively assesses existing or potential gelatinase activity (10). The electrophoretic process also effectively separates MMPs from possible endogenous inhibitors such as TIMPs (10). It is important to note that the proenzyme form can be activated due to reconfiguration by biological molecules in vivo (11, 12, 13) as well as by denaturing conditions in the zymography assay (3, 14).

Briefly, the samples were taken out of the freezer and ground to a powder in a mortar and a pestle. Liquid nitrogen was added to the mortar to keep the samples frozen. Zymography homogenizing buffer (KCl 100mM, $ZnCl_2$ 0.5mM, EDTA 10mM, Tris-HCl 1M, pH 6.8, store at –20°C) was added to the ground samples (approximately 500µl buffer per 10 mg tissue sample), which were sonicated for 30 seconds on ice and then centrifuged at 10000 rpm for 8 minutes at 4°C. The supernatant was collected for protein assay (BCA protein assay kit, Pierce, Rockford, IL). Supernatants containing equal protein amounts (5µg) were prepared by dilution in a 6X sample buffer and loaded into a 7.5% polyacrylamide gel containing SDS copolymerised with 3mg/mL gelatin run at 100 mV for 20 minutes, 150 mV for 40 minutes. Following electrophoresis, the gels were washed in 2.5% Triton X-100 (3 × 20 minutes) at room temperature and incubated in development buffer (0.15M NaCl, 5mM $CaCl_2$, 0.05% NaN_3, 50mM Tris-HCl pH 7.6) for 120 hours at 37°C. The gels were then stained in Coomassie blue for 1.5 hours (1.5g/L Coomassie Brilliant Blue R-250, 25% methanol, 10% acetic acid, 65% water) and destained for 4 hours (4% ethanol, 8% acetic acid, 88% water). The clear bands indicating gelatinase activity were scanned with a densitometer (Fluor-S MAX MultiImager, Bio-Rad Laboratories, CA) and quantitated using the Quantity One Image Analysis System (Bio-Rad Laboratories, CA).

We detected two bands at the sites of 62 kDa and 68 kDa of frog MMP-2 and one band at the site of 88kDa of frog MMP-9 in our zymographies. MMP-2 and MMP-9 were identified by electrophoretic mobility in comparison with a standard (human MMP-2/MMP-9 zymography standard, Chemicon International, Tenecula, CA) run concurrently on the gel. The MMP-2 and MMP-9 signals detected from all samples were within the linear range of our zymography assay (a standard curve was constructed). Because band densities for replicates of the same sample were more variable between different gels than on the same gel, comparisons were always made between samples run on the same gel.

3.2.4 Western blot analysis

Tissue samples were ground to a powder, and Western blot homogenizing buffer (98% zymography homogenizing buffer, 1% protein inhibitor cocktail, 1% DTT) was added and supernatant was collected as described above. Protein concentrations were measured (BCA protein assay kit, Pierce, Rockford, IL). In order to detect TIMP-1 in a frog tissue, we had to prepare samples with a 300μg protein amount. Supernatants containing equal protein amounts (300μg) were reduced and denatured (by boiling for 5 minutes) in a 5X sample buffer (1mL 0.5M Tris-HCl pH 6.8, 1mL β-mercaptoethanol, 4mL glycerol, 2mL 20% SDS, 4 mg bromophenol blue), then loaded on a 10% gel at 60mV for 30 minutes and 140mV for an additional 40-60 minutes in running buffer. Gels were stained with Coomassie blue to verify the protein load. Following electrophoresis, the gel, nitrocellulose membranes and blotting filters were equilibrated for 10 minutes in transfer buffer (9.0g Tris, 43.2g glycine, 600 mL methanol, water added to 3L). Proteins were then transferred electrophoretically using a transfer apparatus (Bio-Rad Laboratories, CA) for 2 hours at 4°C at 70 mV. The nitrocellulose membranes were blocked overnight at 4°C in 5% milk powder diluted in TPBS (PBS with 0.05% Tween). The membranes were incubated in monoclonal primary antibody direct at TIMP-1 (1:250, Calbiochem, San Diego, CA). The primary antibody was detected with secondary antibody using an ECL Chemiluminescence kit (Amersham Pharmacia Biotech, England, UK). Protein bands were compared to a molecular weight marker (Broad-range unstained marker, Bio-Rad Laboratories, CA) that was run concurrently on the gel.

Because there were no anti-frog MMP antibodies available in the market, we had to use mouse against human TIMP-1 monoclonal primary antibody. The cross-reaction effect between the mouse antibody and the frog tissue was very weak; we had to increase the amount of the loading protein and the amount of the primary antibody in order to get distinguishable bands. However, the bans obtained were not sufficient to be quantified. Moreover, although the protein we detected had a molecular weight consistent with TIMP-1, we were unable to confirm that this protein was TIMP-1 in the frog tissue. Although it was not possible to quantify these possibly-TIMP-1-protein levels under this circumstance, qualitative differences between treatments were quite substantial. In our study, we were testing the effect of an herbal drug, as long as the effect of the drug on the model is significant, quantification is not the most important thing to be concerned with.

3.2.5 Statistics

One way ANOVA was used to compare among three groups. Student's t-test was used to compare between two groups. Significance was considered when $p< 0.05$.

3.3 RESULTS

The zymography result of the tissues taken from the fresh un-treated frog palate (n=3) was used as a general control. A sample of zymography gel is shown in **Figure 3.1**. A sample of Western Blot membrane image is shown in **Figure 3.2**.

3.3.1 Comparison of normal and sodium metabisulphite injured tissue for activation of MMP and release of TIMP under different incubations

Because there was much less variability in sample detection by using the same gel for zymography and Western blot analysis (see Material and Methods), we carried out zymographic analyse comparing activation of MMP-9 from FR-incubated control (n=3), FR- incubated MB-treated tissue samples of MB (10^{-2}M) (n=3) and MB (10^{-1}M) (n=3) (**Figure 3.3**). MMP-2 activation was compared in the same fashion from the same gel. TIMP-1-like protein released from FR-incubated control and FR-incubated MB-treated tissue samples was compared on the same Western blot of MB (10^{-1}M) (n=3) and MB (10^{-2}M) (n=3) (**Figure 3.4**).

We detected the pro-forms and the active forms of the MMP-2 (62, 68kDa) and only the active form of MMP-9 (88kDa) in the supernatants of the tissue samples. One way ANOVA confirmed significant difference among the MMP-9 activation of tissue samples from control, MB (10^{-2}M) and MB (10^{-1}M) treatments ($p<0.001$, n=3). Tissues from FR-incubated MB (10^{-1}M) and MB (10^{-2}M) treated palates showed significantly more MMP-9 activation compared to tissues from FR-incubated control palates (486±56%, $p<0.001$, n=3; 89±11%, $p<0.05$, n=3) (**Figure 3.3**). However, there were no significant differences of the MMP-2 activation among the three different treatments.

We detected TIMP-1-like protein (29kDa), in supernatants from tissue samples. TIMP-1-like protein release tended to be reduced, in tissues from FR-incubated MB (10^{-1}M) treated palates compared to tissues from FR-incubated control palates (**Figure 3.4**). In tissues from FR-incubated MB (10^{-2}M) treated palates, this difference was less pronounced; however, there still was a trend indicating decreased TIMP-1-like protein release from FR-incubated MB (10^{-2}M) treated palates (**Figure 3.4**).

We then evaluated the MMP-9 activation from tissues from OP (1mg/ml)-incubated control palates and tissues from OP (1mg/ml)-incubated MB (10^{-2}M) (n=3), MB (10^{-1}M) (n=3) treated palates (**Figure 3.5**). Similarly, the variation of MMP-2 activation was also detected. The release of TIMP-1-like protein from tissues from OP-incubated control palates (n=3), OP-incubated MB (10^{-2}M) treated palates (n=3) and OP-incubated MB (10^{-1}M) treated palates (n=3) (**Figure 3.6**) were compared.

Although pre-incubated in OP overnight, the tissues from OP (1mg/ml)-incubated MB (10^{-1}M) and MB (10^{-2}M) treated palates still were observed to have significantly more MMP-9 activation compared to the tissues from OP (1mg/ml)-incubated control palates (104±18%, $p<0.01$, n=3; 62±5%, $p<0.01$ n=3; **Figure 3.5**). One way ANOVA confirmed the significant difference among the MMP-9 activation of three different treatments ($p<0.001$, n=3). Similar to FR-incubated samples, there was no difference found in MMP-2 activation in the samples with different treatments.

We detected TIMP-1-like protein (29kDa), in supernatants from tissue samples. As FR-incubated samples, a decrease of TIMP-1-like protein release was detected with the increase of MB dose (**Figure 3.6**).

3.3.2 The effect of ophiopogonis incubation on MMP activation and TIMP-like protein release from normal and sodium metabisulphite injured tissue

We next compared MMP-9 activation from tissues from FR-incubated and OP-incubated control palates (n=3), MB (10^{-2}M) treated palates (n=3) and MB (10^{-1}M) treated palates (n=3) (**Figure 3.7A, B, C**). MMP-2 activation of the same gel was also compared. The comparisons of TIMP-like-protein in the same pattern were showed in **Figure 3.8 A, B, C**.

There were no significant effects ($p=0.27$, n=3) of varying FR or OP incubation on MMP-9 activation in the tissues collected from the control palates (**Figure 3.7A**). MMP-

9 activation was significantly reduced in tissues from OP (1mg/ml)-incubated MB (10^{-2}M) treated palates compared to FR-incubated MB (10^{-2}M) treated palates (59±7%, $p<0.01$, $n=3$) (**Figure 3.7B**). MMP-9 activation was also significantly reduced in tissues from OP (1mg/ml)-incubated MB (10^{-1}M) treated palates compared to FR-incubated MB (10^{-1}M) treated palates (438±39%, $p<0.01$, $n=3$) (**Figure 3.7C**). No significant change was found in the activation of MMP-2.

There were no apparent changes in TIMP-1-like protein release in the tissues from OP or FR-incubated control palates (**Figure 3.8A**). TIMP-1-like protein release appeared to be was reduced in the tissues from OP (1mg/ml)-incubated MB (10^{-2}M) treated palates (**Figure 3.8B**) and also appeared to be decreased in the tissues from OP (1mg/ml)-incubated MB (10^{-1}M) treated palates (**Figure 3.8C**).

3.3.3 Results of SEM study

In **Figure 3.9A-D**, examples of the surface of frog palate (×400) are shown under four conditions: **Figure 3.9A** shows the tissue collected from FR-incubated control palate. The cilia appear as uniform fine dots covering the entire surface of the palate, interspersed with pores. **Figure 3.9B** shows the tissue collected from FR-incubated MB (10^{-1}M) treated palate. Under this condition, the surface of the palate appears to exhibit areas of deciliation (i.e. bare areas), interspersed among ciliated areas. **Figure 3.9C** shows the tissue collected from an OP (1mg/ml)-incubated control palate. In this view, the palate surface appears similar to **Figure 3.9A,** indicating that incubation in OP has no effect on cilia. **Figure 3.9D** shows the tissue collected from an OP (1mg/ml)-incubated MB (10^{-1}M) treated palate. The ciliated surface of this palate appears normal, indicating that in the presence of OP, deciliation by MB was prevented.

In **Figure 3.10** quantification of the covering areas of cilia was compared. It shows the cilia covering area difference between FR-incubated, MB (10^{-1}M) treated tissue and FR-incubated control; significant cilia loss (~20±10%, $p<0.01$, $n=3$) was apparent in the injured tissue.

3.4 DISCUSSION

Previous studies showed that MB could induce an acute epithelial injury (15). Studies of SEM revealed that MB caused sloughing of the ciliated epithelial cells on the palate, which would interfere with mucociliary clearance. In the normal tissue from the FR-incubated control palate (**Figure 3.9A**) the cilia appear as uniform fine dots covering the entire surface of the palate, interspersed with pores. After the application of MB (10^{-1}M) (**Figure 3.9B**), the surface of the palate appears to have areas of deciliation (i.e. bare areas), interspersed among ciliated areas, indicating the sloughing of the cilia or the ciliated epithelial cells.

MMPs are expressed at low levels in normal tissues, and their upregulation appears to play an important role in the development of a number of pathological processes (16). Accumulating evidence indicates the importance of MMPs and TIMPs in lung development as well as in a variety of pulmonary inflammatory disorders, including asthma (17), COPD (18), emphysema (19) and acute lung injury (20). The expressive balance between proteases and protease inhibitors plays a critical role in maintaining the degradation and synthesis of extracellular matrix (16). The loss of this balance is associated with airway injury (21) and remodeling (22). In our studies, tissues from FR-incubated MB (10^{-1}M) treated frog palates demonstrated significantly increased MMP-9 and decreased TIMP-1 release (**Figure 3.3, Figure 3.4**) while no significant changes were detected in the level of MMP-2 activation. These results suggest that the epithelial injury observed in the MB induced injury may be due in part to increased levels of ECM-remodeling enzymes. The MMP-9 we detected was the active form of 88kDa. This indicated that the MMP-9 was already activated and freed from the MMP-9/TIMP-1 complex and was released into the extracellular environment, and thus may have directly contributed to the tissue damage. Furthermore, for the tissues from FR-incubated MB (10^{-2}M) treated frog palates, neither MMP-9 increases (**Figure 3.3**) nor TIMP-1 decreases (**Figure 3.4**) in as marked a manner as the response to MB (10^{-1}M). This suggested that the extent of injury and tissue damage caused by MB (10^{-2}M) was not as severe as that in response to MB (10^{-1}M) exposure.

Altered MMP-9 levels may be due to many factors, one of which possibly is the elevated production of nitric oxide (NO). Elevated MMP-9 levels in inflammatory lung diseases are thought to originate from infiltrating granulocytes or alveolar macrophages. However, human bronchial epithelial cells are an important source of MMPs (primarily MMP-2 and MMP-9) in the lung (25). Although epithelial cells constitutively express MMP-2, MMP-

9 expression can be induced by various proinflammatory cytokines, growth factors, and LPS (26) through activation of the transcription factors nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) (27, 28). Inflammatory conditions of the respiratory tract are also commonly characterized by an elevated production of NO through increased expression of inducible NO synthase (iNOS) within the respiratory epithelium and in inflammatory cells, such as monocytes/macrophages and neutrophils (29, 30, 31). Several recent studies have identified various regulatory properties of NO and/or reactive nitrogen species (RNS) on gene expression and activation of MMPs. For instance, several RNS are capable of activating MMPs, including human neutrophil collagenase (MMP-8) and MMP-9 (12, 32), via oxidative modification of the cysteine switch. In addition, $NO^{\cdot}$ has been reported to induce MMP-9 expression in chondrocytes (34) but was found to downregulate MMP-9 expression in mesangial cells (35) and aortic smooth muscle cells (33, 36).

The significantly decreased MMP-9 and increased TIMP-1-like protein in the tissue from OP (1mg/ml)-incubated MB (10^{-1}M) treated frog palates compared to those incubated in FR and treated in the same way (**Figure 3.7C, Figure 3.8C**) suggested that the herb OP, as a natural product, had beneficial effects on injured epithelial tissue. The protective effect of OP was more apparent by SEM studies. Tissue from palate incubated in OR overnight prior to the application of MB (**Figure 3.9D**) showed much less cell sloughing, which produces a corresponding beneficial effect on the recovery of mucociliary clearance function, as described in Chapter 2. These studies suggest that MB affects by reducing the otherwise continuous carpet of cilia on the normal frog palate over which mucus flows (23). While many factors affect the flow of mucus on the palate, a continuous cilia bed is vital for the efficient movement of mucus over the palate. An interruption would be expected to have significant effects on mucus transport and clearance (24). Our findings suggest that OP reduces the amount of cell sloughing, and preserves the mucociliary function by keeping the cilia bed in continuous condition, thus, helping to inhibit the injury to the epithelial tissue. Since there was also a significant increase in MMP-9 activation and decrease in TIMP-1 release in tissues from OP (1mg/ml)-incubated MB (10^{-1}M) frog palates compared with tissues from OP (1mg/ml)-incubated control palates, this further suggests that OP can only partially inhibits MB-mediated epithelial injury.

In all these studies, it was shown that OP incubation had no perceptible effect that differed from the FR-incubation control groups. These results suggest that OP has much less potentially beneficial effects on normal epithelium compared with those seen in abnormal epithelium.

The animal model we used were bullfrogs; since there were no anti-frog MMP antibodies available on the market, we had to use a mouse monoclonal antibody. The cross-reaction effect between the mouse antibody and the frog tissue was very weak. The bands we have got can tell the differences between the samples but not qualified to be quantified. Moreover, although the protein we detected had a consistent molecular weight with TIMP-1, we still could not confirm that this protein was TIMP-1 in the frog tissue.

Similarly, since few researches has been done to investigate the molecular weight of frog MMPs, the isoforms of MMPs we obtained are not necessarily frog MMP-2 and MMP-9. In conclusion, we measured activation of MMPs and a possible inhibitor from tissues from control or MB-injured tissue under the FR or OP incubation. We demonstrated increased activation of MMP-9, decreased release of TIMP-1-like protein and no significant changes in MMP-2 in the tissues from FR-incubated MB treated frog palates compared to FR-incubated control. These results suggest that epithelial injury occurs as a result of MB induction; this result is also supported by the SEM studies. We also observed the tissues that are less responsive to changes in the OP (1mg/ml)-incubated frog palates, reflected in our study by MMP-9 activation and TIMP-1-like protein release. We suggest that tissues from OP-incubation have a diminished capacity to response to MB injured frog palates, which may indicate the beneficial protective effect that OP has on the injured epithelium.

3.5 REFERENCES

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Example of Zymography Gel

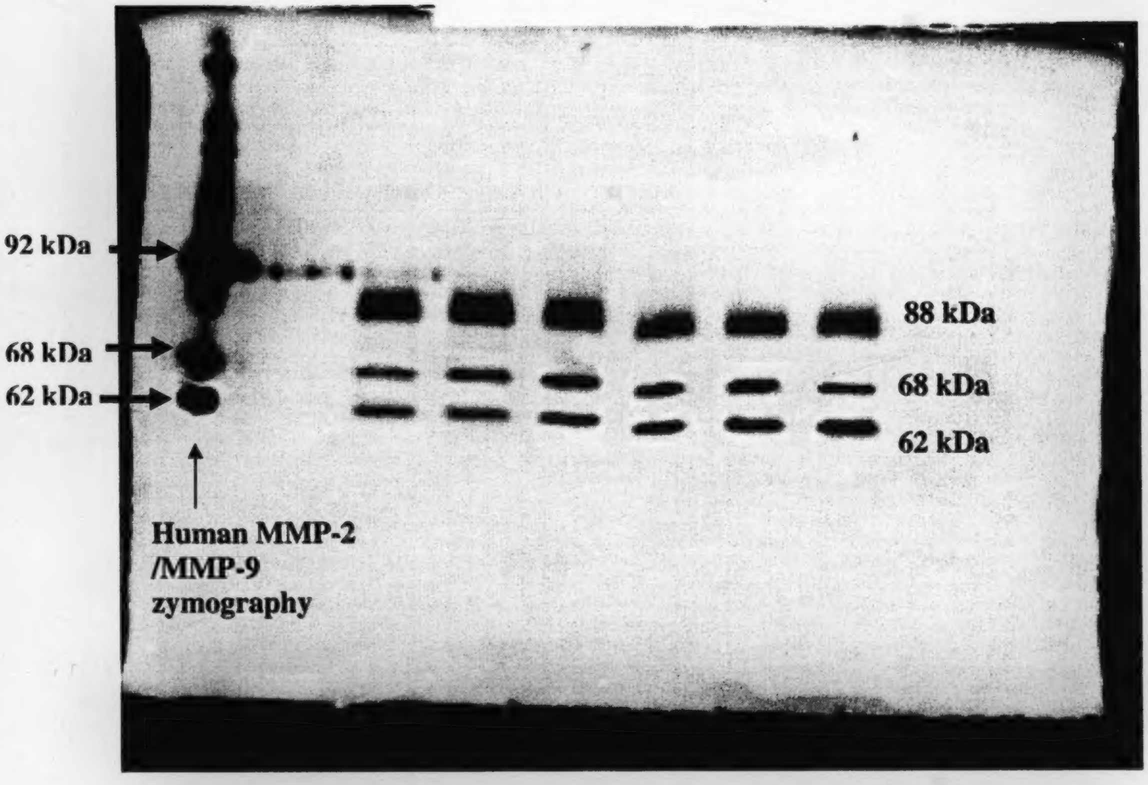


Figure 3.1 Zymography gel example.

Example of Western Blot Membrane

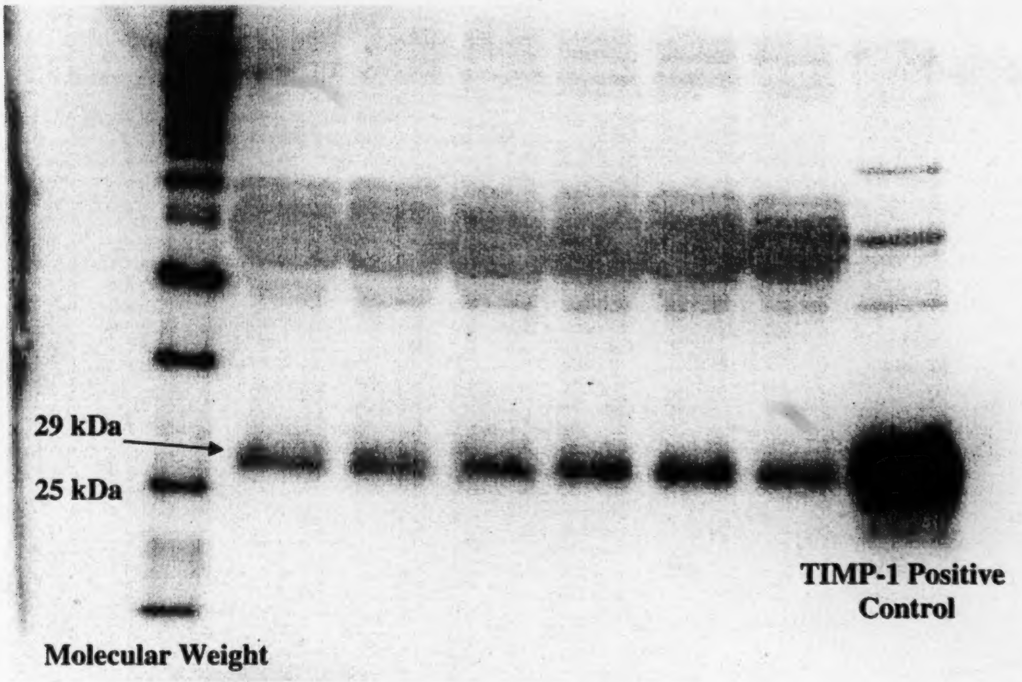


Figure 3.2 Western Blot membrane image example.

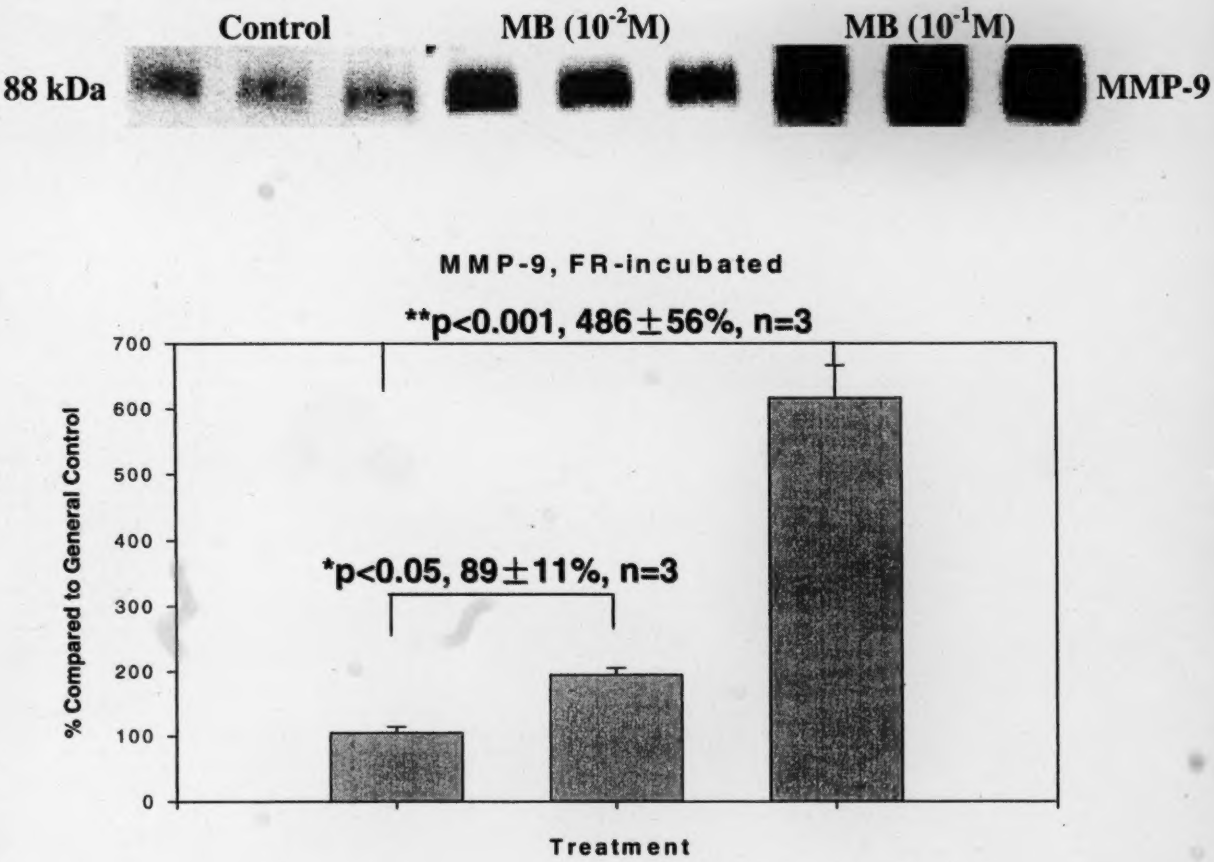


Figure 3.3 Comparison for MMP-9 activation of normal and MB injured tissue under FR-incubation.

Zymography analysis of MMP-9 activation FR-incubated frog palate tissue (n=3, control; n=3, MB (10^{-2}M); n=3, MB (10^{-1}M)). The upper panels show the zymogram (5 µg of protein per lane) of MMP-9 activation. The lower panels show summary data as percent compared to the general control (5µg of protein from the untreated tissue). The data are presented as means ± SEM of percent compared to the control. ** $p< 0.001$ for MMP-9 activation of MB (10^{-1}M) treated tissues compared to control. * $p< 0.05$ for MMP-9 activation of MB (10^{-2}M) treated tissues.

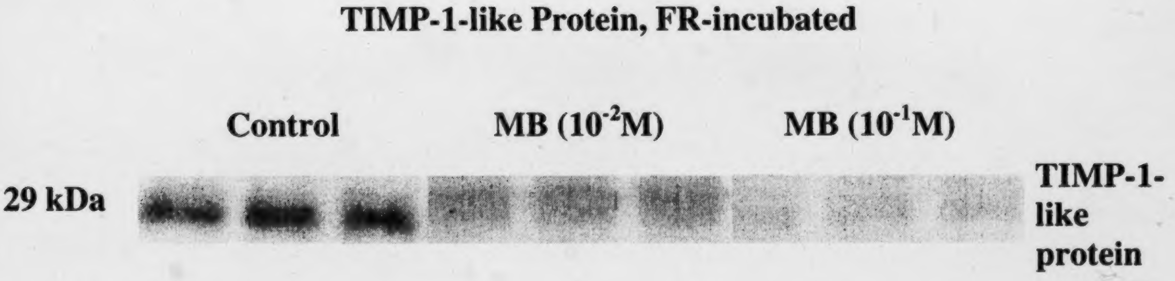


Figure 3.4 Comparison for release of TIMP-1-like protein of normal and MB injured tissue under FR-incubation.

Western blot analysis of TIMP-1-like protein release from tissues from FR-incubated control and MB-treated palates (n=3, control; n=3, MB (10^{-2}M); n=3, MB (10^{-1}M)). The panels show the Western blot (300 μg of protein per lane) of TIMP-1-like protein release. No densitometry data were shown because some of the bands were too weak and the borders were too vague to be quantified.

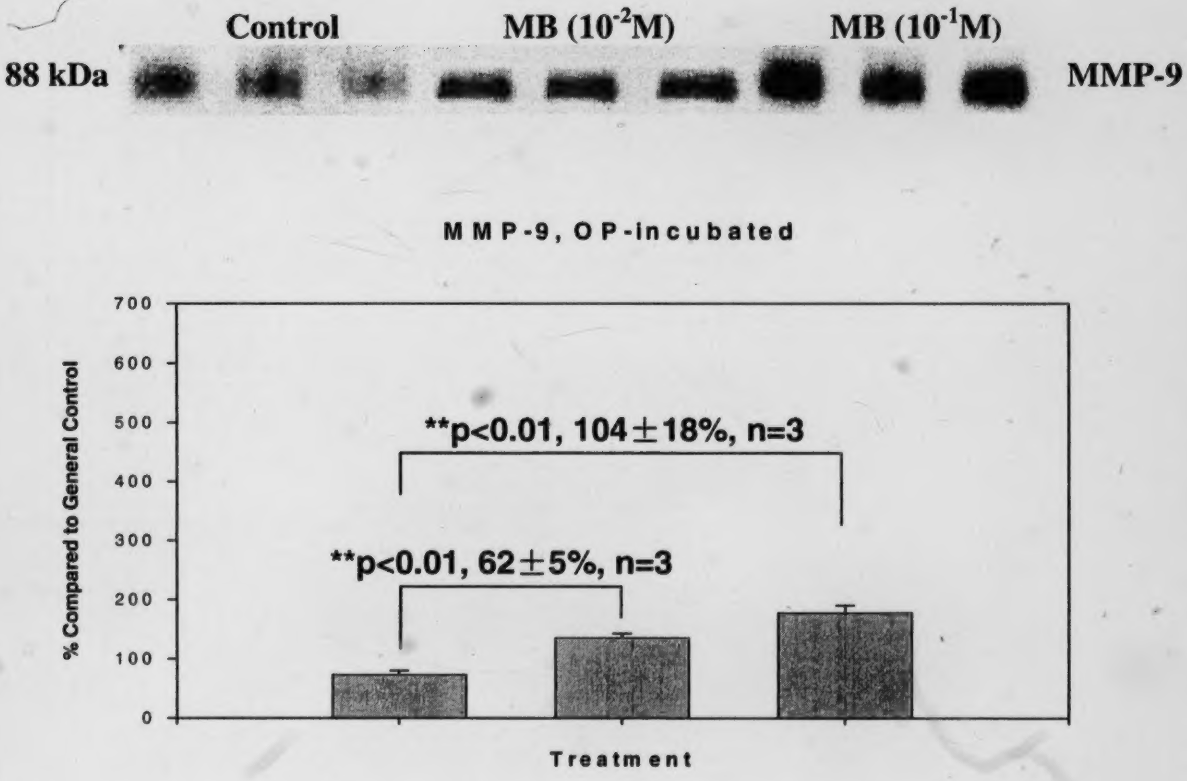


Figure 3.5 Comparison for MMP-9 activation of normal and MB injured tissue under OP-incubation.

Zymography analysis of MMP-9 activation from palates pre-incubated in OP (overnight), (n=3, control; n=3, MB (10^{-2}M) treated; n=3, MB (10^{-1}M) treated). The upper panels show the zymogram (5 μg of protein per lane) of MMP-9 activation. The lower panels show summary data as percent compared to the general control (5 μg of protein from untreated tissue). The data are presented as means ± SEM of the percent compared to the control. **p< 0.01 for tissues treated by MB (10^{-1}M) and MB (10^{-2}M) for MMP-9 activation compared to control.

TIMP-1-like Protein, OP-incubated

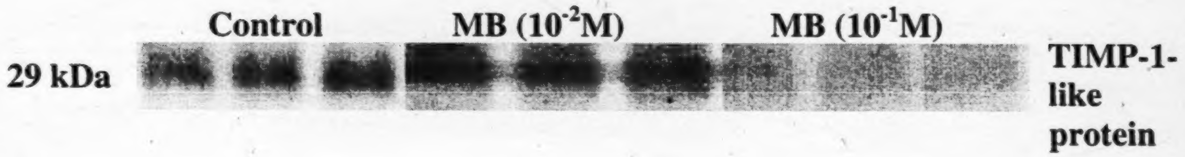
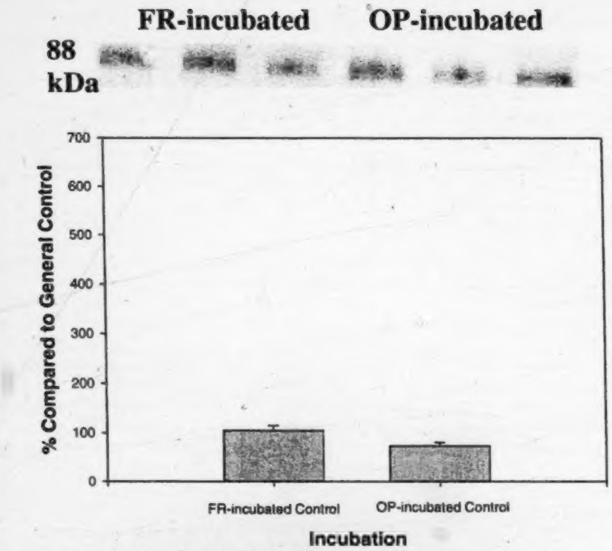
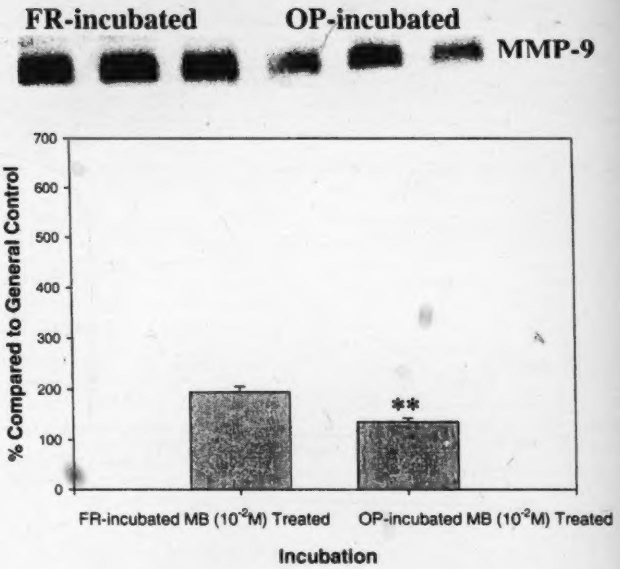


Figure 3.6 Comparison for release of TIMP-1-like protein of normal and MB injured tissue under OP-incubation.

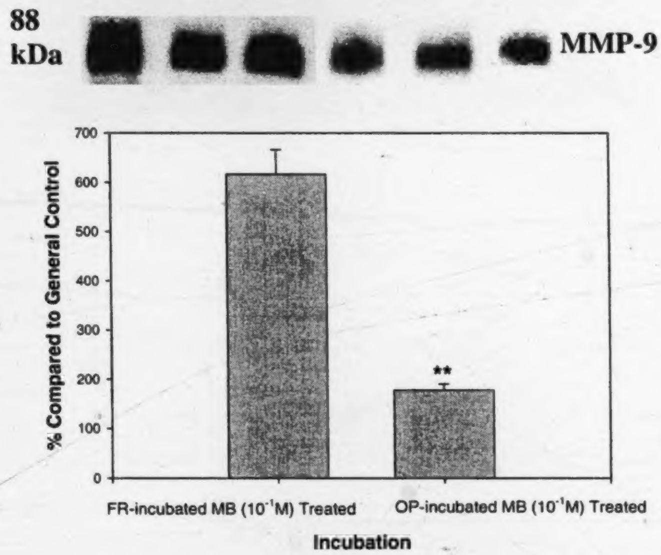
Western blot analysis of TIMP-1-like protein release from tissues from OP-incubated control and MB-treated palates (n=3, control; n=3, MB (10^{-2}M); n=3, MB (10^{-1}M)). The panels show the Western blot (300 µg of protein per lane) of TIMP-1-like protein release. No densitometry data were shown because some of the bands were too weak and the borders were too vague to be quantified.



(A) No significant difference, n=3



(B) **p<0.01, 59±7%, n=3



(C) **p<0.001, 438±39%, n=3

Figure 3.7 The effect of OP-incubation on MMP-9 activation for normal and MB injured tissue.

MMP-9 activation of the tissue from control pre-incubated (overnight) in FR or OP (n=3, FR; n=3, OP; **A**). Also shown is MMP-9 activation of the tissue treated by MB (10^{-2}M), pre-incubated (overnight) in FR or OP (n=3, FR; n=3, OP; **B**); tissue treated by MB (10^{-1}M) pre-incubated in FR or OP (n=3, FR; n=3, OP; **C**). The upper panels show the zymogram (5 μg of protein per lane) of MMP-9 activation. The lower panels show summary data as percent compared to the general control (5 μg of protein from untreated tissue). The data are presented as means ± SEM of the percent compared to the control. ** $p<0.001$ for MB (10^{-2}M) and MB (10^{-1}M) treatment.

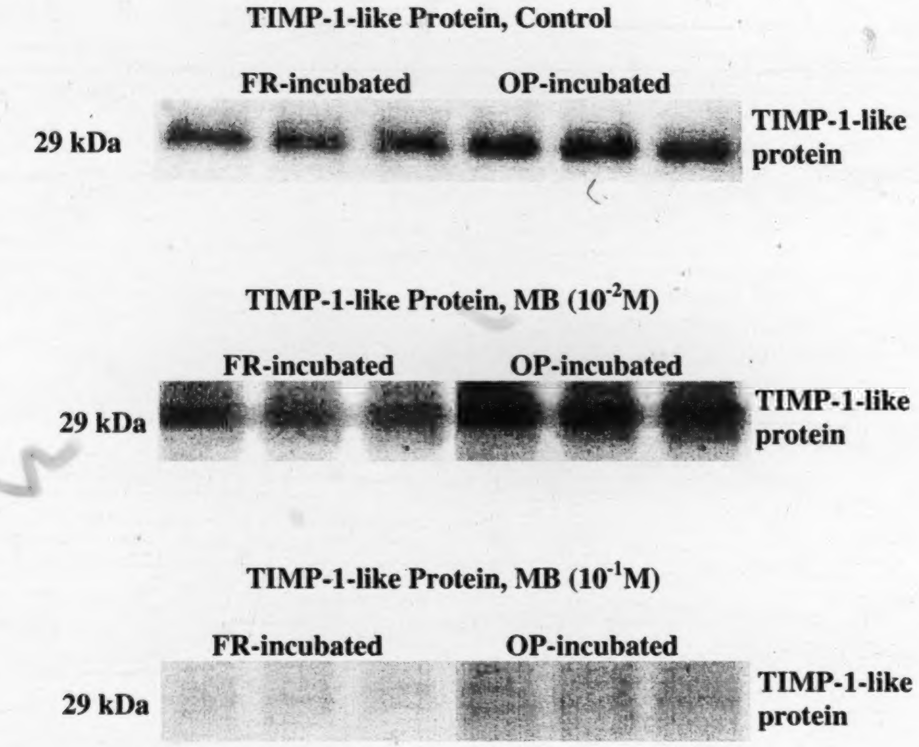


Figure 3.8 The effect of OP-incubation on TIMP-1-like protein release for normal and MB injured tissue.

Western blot analysis of TIMP-1-like protein release from tissues from control (n=3, FR-incubated; n=3, OP-incubated; **A**), MB (10^{-2}M) treated (n=3, FR-incubated; n=3, OP-incubated; **B**) and MB (10^{-1}M) treated (n=3, FR-incubated; n=3, OP-incubated; **C**). The panels show the Western blot (300 µg of protein per lane) of TIMP-1-like protein release. No densitometry data were shown because some of the bands were too weak and the borders were too vague to be quantified.

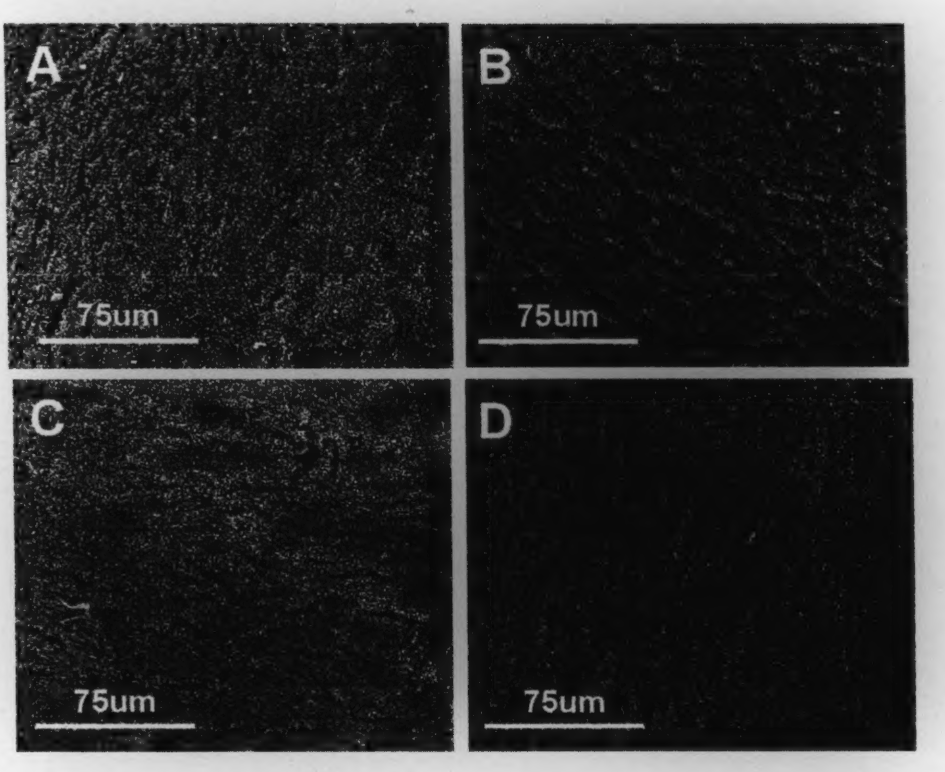


Figure 3.9 Scanning electron micrographs (SEM) of the epithelial surface of frog palates (×3500) FR/OP pre-incubated and with/without MB (10^{-1}M) treatment.

Figure 3.9A shows the tissue collected from FR-incubated (overnight) control palate. The cilia appear as uniform fine dots covering the entire surface of the palate, interspersed with pores. **Figure 3.9B** shows the tissue collected from FR-incubated (overnight) MB (10^{-1}M) treated palate. Under this condition, the surface of the palate appears to exhibit areas of deciliation (i.e. bare areas), interspersed among ciliated areas. **Figure 3.9C** shows the tissue collected from an OP (1mg/ml)-incubated (overnight) control palate. **Figure 3.9D** shows the tissue collected from an OP (1mg/ml)-incubated (overnight) MB (10^{-1}M) treated palate. The ciliated surface of this palate appears normal, indicating that in the presence of OP, deciliation by MB was prevented.

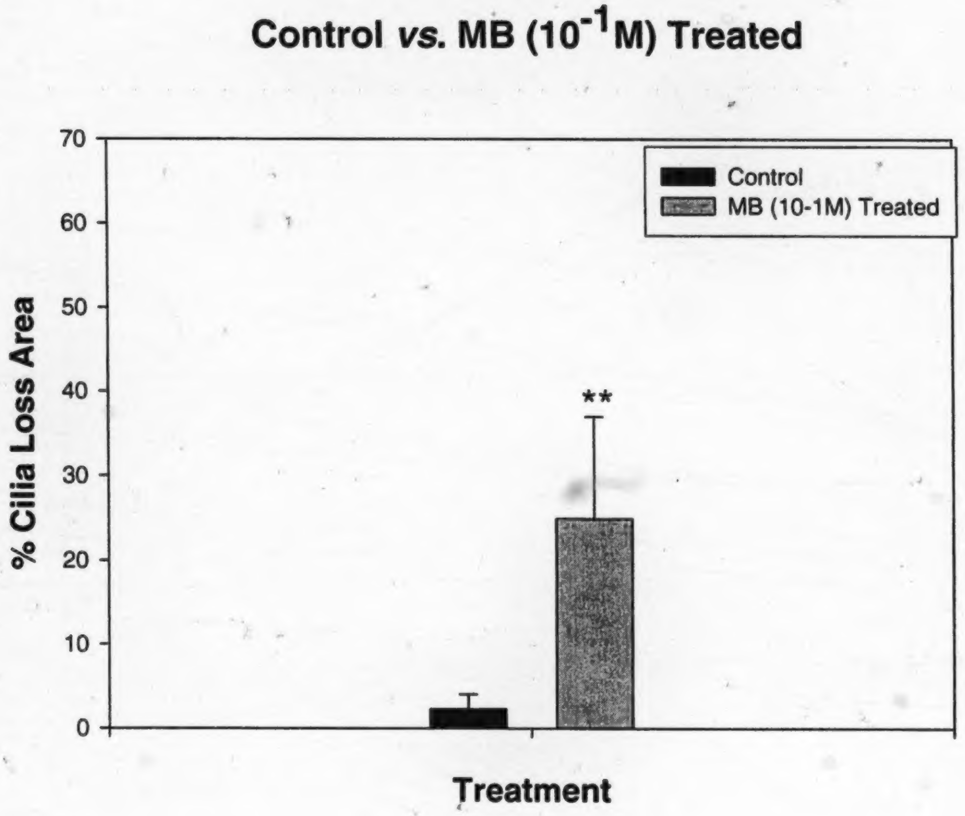


Figure 3.10 Comparison of cilia loss area between the FR-incubated control frog palate tissue and the FR-incubated MB (10^{-1}M) treated frog palate tissue.

Areas of cilia loss were measured from the result of SEM studies. Percent of cilia loss area compared to the whole SEM studying area of the FR-incubated control (n=3) and FR-incubated MB (10^{-1}M) treated frog palate tissue (n=3) were compared. ** $p < 0.001$ of the MB (10^{-1}M) treated tissue compared to control for cilia loss area.

CHAPTER 4. THE ROLE OF MATRIX METALLOPROTEINASES IN CIGARETTE SMOKE INDUCED EPITHELIAL INJURY.

4.1 INTRODUCTION

Cigarette smoke has the capacity to damage the airway in a number of ways, including direct toxicity to the airway epithelium (1), oxidative damage (2), recruitment of inflammatory cells (3), and increased epithelial permeability (4). In the absence of atopy, smoking is related to bronchial hyperresponsiveness in nonasthmatics, and results in airway inflammation (3).

Once inflammation is triggered by cigarette smoke, it will cause damage in the airways. A large number of processes are involved including damage to the cilia (5). When cilia are injured, pathogenic agents become trapped in the lungs and can cause infections that lead to chronic bronchitis (6).

Cigarette smoke is a complex mixture of > 4,700 chemical compounds of which free radicals and other oxidants are present in high concentrations. The gas phase of cigarette smoke contains approximately 10^{15} radicals per puff, primarily of the alkyl and peroxyl types, and 10^{20} oxidant molecules per puff in total (7). These bring an excessive oxidant burden to the respiratory system, which may lead to oxidative stress (8). Oxidative stress includes inactivation of antiproteinases, epithelial injury, increased sequestration of neutrophils in the pulmonary microvasculature, and enhanced expression of proinflammatory mediators (2)

Proteases are enzymes released by many cell types including neutrophils, mast cells, endothelial cells and epithelial cells (10). Under normal circumstances these immune factors are important for fighting infection and injury. However, smoking can incite the immune system to the extent that proteases become overproduced. In excess, these enzymes can damage the structure of the airways and the lung parenchyma (11). Antiproteases are the proteins that ordinarily neutralize the proteases. When cigarette smoke is inhaled and the airway is exposed to the smoke, a protease/antiprotease imbalance is induced (12).

MMPs are one group of protease that may be involved in cigarette smoke-induced airway epithelial injury. Investigators have demonstrated numerous effects of long term cigarette smoke exposure on the release of MMPs and their inhibitors (TIMPs) in various cell types. Increased MMP-9 levels and decreased TIMP-1 levels were found in the alveolar macrophages of heavy-smokers when compared to nonsmokers (13). In rat lungs, exposure to cigarette smoke induced increases in TIMP-1 mRNA levels (14). Upregulation of MMP-9 gene has also been reported when human vascular endothelial cells were exposed to cigarette smoke condensate (15). It also has been reported that cigarette smoke exposure induced increase in the expression of MMP-2 protein expression in the lung of rat (14). Despite all the above studies, however, the acute effect of cigarette smoke exposure on epithelial cells has not been well characterized.

In our study, we observed the physiological changes in the ciliated epithelium and tested the mucociliary clearance function after acute smoke exposure. We hypothesized that MMPs may play an important role in the process leading to airway epithelial damage.

4.2 MATERIAL AND METHODS

4.2.1 Animals and Experimental protocol

From a bullfrog, *Rana catesbiana*, the upper portion of the head was removed, modifying the procedures described in previous work (23, 24), by cutting with scissors through from the junction of the posterior pharynx and esophagus out to the skin of the back. The excised palate was then inverted and placed immediately onto a petri dish with a piece of gauze soaked in FR. The palate was placed in the middle of the mucociliary clearance observation chamber, as previously described.

4.2.2 Mucociliary clearance observation

Mucociliary clearance observation chamber

After the frog palate was isolated, it was placed in the middle of an acrylic chamber (see **Figure 2.1**, 20cm height, 30cm width, 20cm depth) maintained at a constant temperature (22°C to 24°C), which was continuously humidified with FR solution aerosol. Mucociliary clearance movement was detected through a dissecting microscope placed above the chamber.

MCT measurements

As described in Chapter 2, normal mucociliary clearance time (MCT) was measured by applying a droplet of FR to the frog palate. The volume of the FR applied was proportional to the total epithelial area of this palate, usually 2μl to 5μl. Two minutes was allowed to elapse for the solution to be distributed over the palate before measurements. To observe clearance, a drop of clear mucus (diameter 1-2mm) that had been collected from the palate was put on the top of the palate surface (near the mouth side). Once the mucus transport reached a steady rate, MCT was determined by recording the time for the displacement of mucus for 5mm. At least 5 MCT measurements were recorded to arrive at a mean MCT after each treatment.

4.2.3 Frog palate exposure model preparation

Animal preparation

The palate was divided longitudinally into halves along the midline, cutting the epithelium with a scalpel to minimize the damage. After five minutes MCT was measured in both halves, to confirm that the cilia of both half palates were functioning normally. One side was used as control and the other half was exposed to cigarette smoke. The control half of the palate was left in the mucociliary clearance observation chamber while the other half was placed in the exposure chamber, at 100% humidity and room temperature.

Cigarette smoke exposure chamber

The exposure chamber (**Figure 4.1**) with a volume of 10L has two inlets: one connected to an aci-driven Pari jet nebuliser system set at 8L/minute measured by a Puritan flow meter; the other inlet was linked to a cigarette combustion chamber. The combustion chamber, which contained a burning cigarette, was slightly pressurized with air flowing into the chamber at a rate of 2L/minute and ventilated to promote cigarette combustion. Positive ventilation inside the burning chamber pushed the side stream smoke into the covered but not sealed exposure chamber, which was exhausted into a fume hood. The temperature inside the chamber was monitored with a thermocouple and digital-display thermometer. The palate was positioned with the palate side up on a piece of gauze saturated with FR in a petri dish at ~five centimetres above the bottom. We used filtered cigarettes of randomly selected brands that were regularly available at commercial outlets. It took one cigarette 17 minutes on average to burn completely in this preparation.

4.2.4 Tissue collection

After the four-cigarette-smoke-exposure was completed, the epithelial tissue was carefully separated from the palate musculature. A piece of tissue (about 5mm × 15mm) was sectioned from the central part of each half palate, frozen in liquid nitrogen immediately and stored at -80^{o}C for zymography studies.

Scanning Electron Microscope (SEM) Studies

As described in Chapter 3, an additional small piece of epithelial tissue (about 2mm × 8mm) was also taken from each half palate. This tissue sample was placed in 2.5% glutaraldehyde solution immediately after collection and stored in a refrigerator at 4°C until processing. The sample was post-fixed at room temperature for one hour, followed by being washed briefly in distilled water and dehydrated in a series of ethanol (50-100%). The sample was mounted on a specimen holder for SEM and dried overnight; after sputter coating with gold, the sample was examined with a Hitachi S-2500 scanning electron microscope. Digital images were acquired.

4.2.5 Gelatin zymography

Tissue samples were collected as described above and protein concentrations were measured. The MMP-2 and MMP-9 levels in the epithelial tissues were evaluated by zymography according to established methods as described in Chapter 3 (9, 25, 26, 27, 28, 29, 30, 31, 32).

We detected a band at the site of 62kDa of MMP-2 and a band at the site of 88kDa of MMP-9 in our zymographies. MMP-2 and MMP-9s were identified and detected as described in Chapter 3.

4.2.6 Statistics

A Student's t-test was used to compare between two groups. Significance was considered when $p < 0.05$.

4.3 RESULTS

4.3.1 The effects of cigarette smoke exposure on mucociliary clearance function

After the exposure to the smoke from four cigarettes, MCT was significantly increased by (~300%, $P<0.001$, n=3) compared to control (**Figure 4.2**). No difference in MCT was found in the control half palates over the same elapsed time.

4.3.2 The effects of cigarette smoke exposure on MMP activation

The zymography results of the tissues taken from fresh untreated frog palates (n=3) were used as a general control.

We carried out zymographic analyses comparing activation of MMP-9 and MMP-2 from tissues collected from the control half (n=3) of the palate and tissues collected from the half palate exposed to four cigarettes (n=3) (**Figure 4.3A, B**).

We detected MMP-2 (62kDa) and MMP-9 (88kDa) in the supernatants of the tissue samples. Tissues from the half palate exposed to four cigarettes were demonstrated to show significantly more MMP-9 activation compared to the tissues collected from the control half (546±49%, $p<0.001$, $n=3$; **Figure 4.3A**). However, exposure to cigarette smoke did not appear to result in significant changes in the MMP-2 activation ($p=0.096$, $n=3$; **Figure 4.3B**).

4.3.3 Results of SEM study

In **Figure 4.4 A, B**, examples of the surface of frog palate (×400) are shown under two conditions: **Figure 4.4A** shows the tissue collected from the control half of the palate. The cilia appear as uniform fine dots covering the entire surface of the palate, interspersed with pores. **Figure 4.4B** shows the tissue collected from the half of the palate exposed to the smoke from four cigarettes. Large bare areas were detected, indicating a substantial loss of ciliated epithelial cells.

Figure 4.5 shows the quantification of the loss of ciliated surface area between the tissues collected from the half of the palate exposed to four cigarettes smoke and the control half. Significant cilia loss (~50±12%, $p<0.01$, $n=3$) was indicated in the injured tissue.

4.4 DISCUSSION

The frog palate exposure model presents an isolated epithelial system free of interference from other agents or systemic physiological responses resulting from other internal or external influences. In this injury model we randomly exposed either half of the palate to side stream cigarette smoke and used the other half palate as internal control. It has been tested that there was no statistical difference in mucociliary clearance function (MCF) between the two half palates. Moreover, no difference in MCF was found in the control half of palate between the beginning and the end of the experiment.

Mucociliary clearance is a vital pulmonary defence mechanism. The maintenance of this function greatly depends on cilia integrity and performance (16). In our mucociliary studies, we have demonstrated that the MCT of the half palate exposed to four cigarettes smoke was significantly increased compared to the control half, which indicates impaired mucociliary clearance function after smoke exposure. This result suggests that acute cigarette smoke exposure induces epithelial injury and deficiency of the mucociliary clearance system.

In our SEM studies, it also has been found that for the half palate exposed to 4-cigarette smoke (**Figure 4.4B**), large bare areas were detected on the palate surface indicating a great loss of the ciliated epithelial cells; at the same time, the control half palates appeared to have very healthy and intact cilia covering the surface. It is well known that it is the cilia beating that transports the mucus to make the whole mucociliary system work

(17). When palate epithelium was injured and ciliated cells sloughed, the areas left with no cilia on it would have minimal mucociliary clearance ability. We detected both loss of cilia and impaired clearance function; this suggested cigarette smoke induced epithelial injury and mucociliary clearance impairment largely depend on the loss of cilia.

Chronic exposure to cigarette smoke induces an influx of inflammatory cells into the respiratory tract (18). Increased numbers of neutrophils and monocytes, activated by cigarette smoke, produce large amounts of proteases and oxidants (19, 20). Excessive proteases can drive the breakdown of connective tissue. Cigarette smoke can also inactivate antiprotease protection (21). Most of the injury induced by cigarette smoke is due to the imbalance of protease and anti-protease. Thus, the cigarette smoke may influence both matrix damage and repair processes, leading to airway destruction by inflammatory processes (22).

MMPs are a kind of protease important to both airway injury and remodeling. They can be released by inflammatory cells (neutrophils, eosinophils), endothelial cells and epithelial cells. In our studies, we found that the MMP-9 was dramatically increased after the four cigarettes smoke compared to the control (**Figure 4.3 A**). However, the cigarette smoke exposure did not seem to affect the release of MMP-2 very much (**Figure 4.3B**). This suggests that the tissue injury of frog palate ciliated epithelium induced by acute cigarette smoke exposure is more likely due to MMP-9 rather than MMP-2. Similarly, Kang and coworkers demonstrated that in MMP-9 expression was elevated in the lung parenchyma compared with non-smokers (14). Few changes of MMP-2 expression of the cigarette smoke exposure to the mammalians have been reported.

The study was carried out in an ex-vivo model; therefore, the circulation did not exist when we exposed the palates to cigarette smoke. Under these circumstances, it is unlikely for neutrophils to be a major source of MMP-9. This suggests that the excess MMP-9 we have detected may derive from a source other than neutrophils, most likely from the epithelial cells themselves, given the rapidity of the response.

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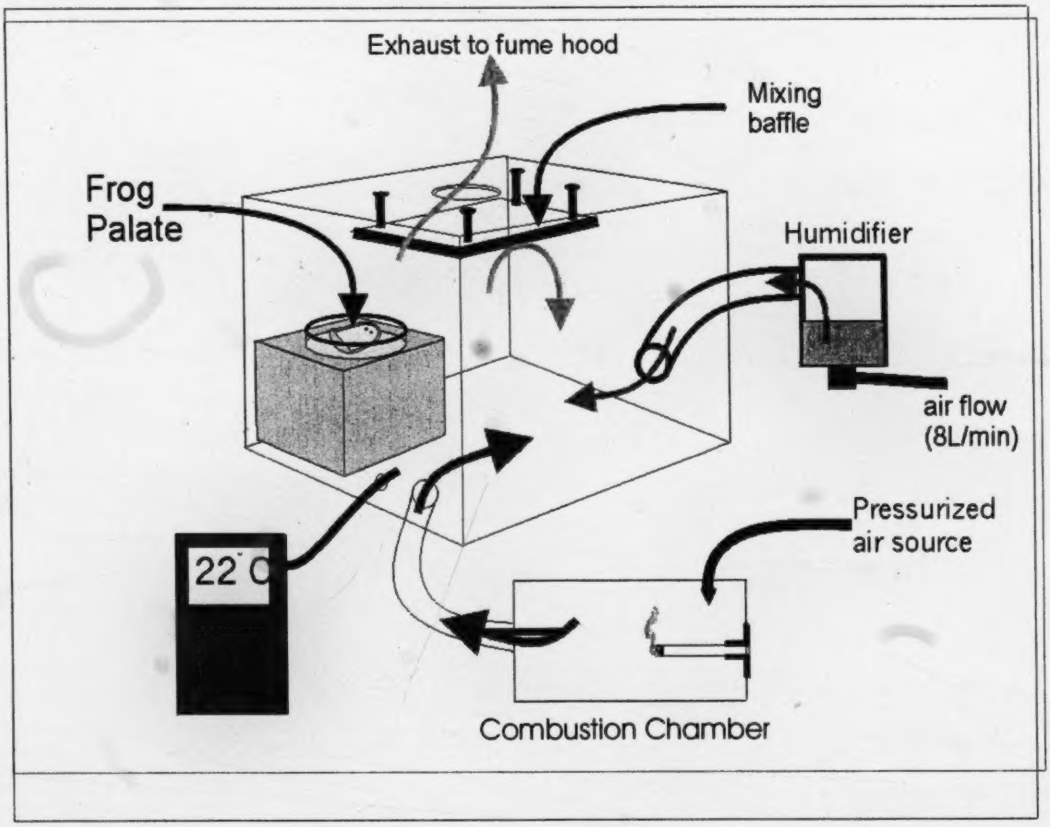


Figure 4.1 Cigarette smoke exposure chamber

This diagram shows a ten-liter chamber with two inlets: one connected to an air-driven Pari jet nebuliser system set at 8L/minute, measured by a Puritan flow meter. The other inlet was linked to a cigarette combustion chamber. The combustion chamber, which contained a burning filtered cigarette, was slightly pressurized with air flowing into the chamber at a rate of 2L/minute and ventilated to promote cigarette combustion. The temperature inside the chamber was monitored with a thermocouple and digital-display thermometer. The excised palate was placed with the epithelium side up on a piece of gauze saturated with FR in a petri dish.

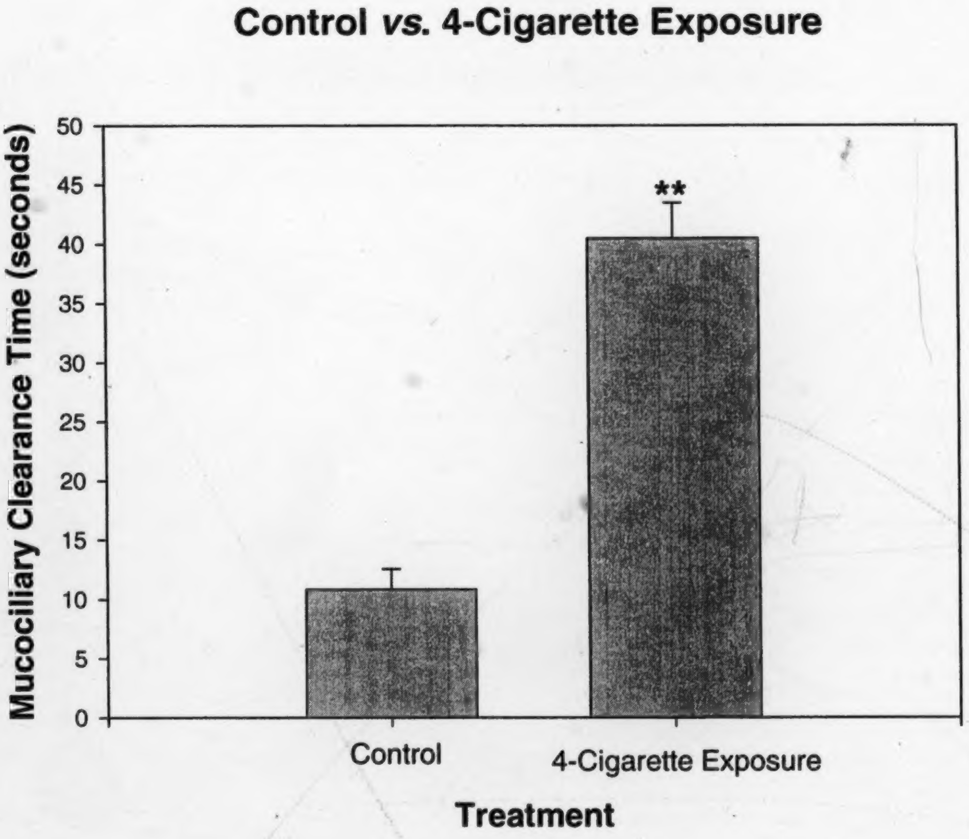
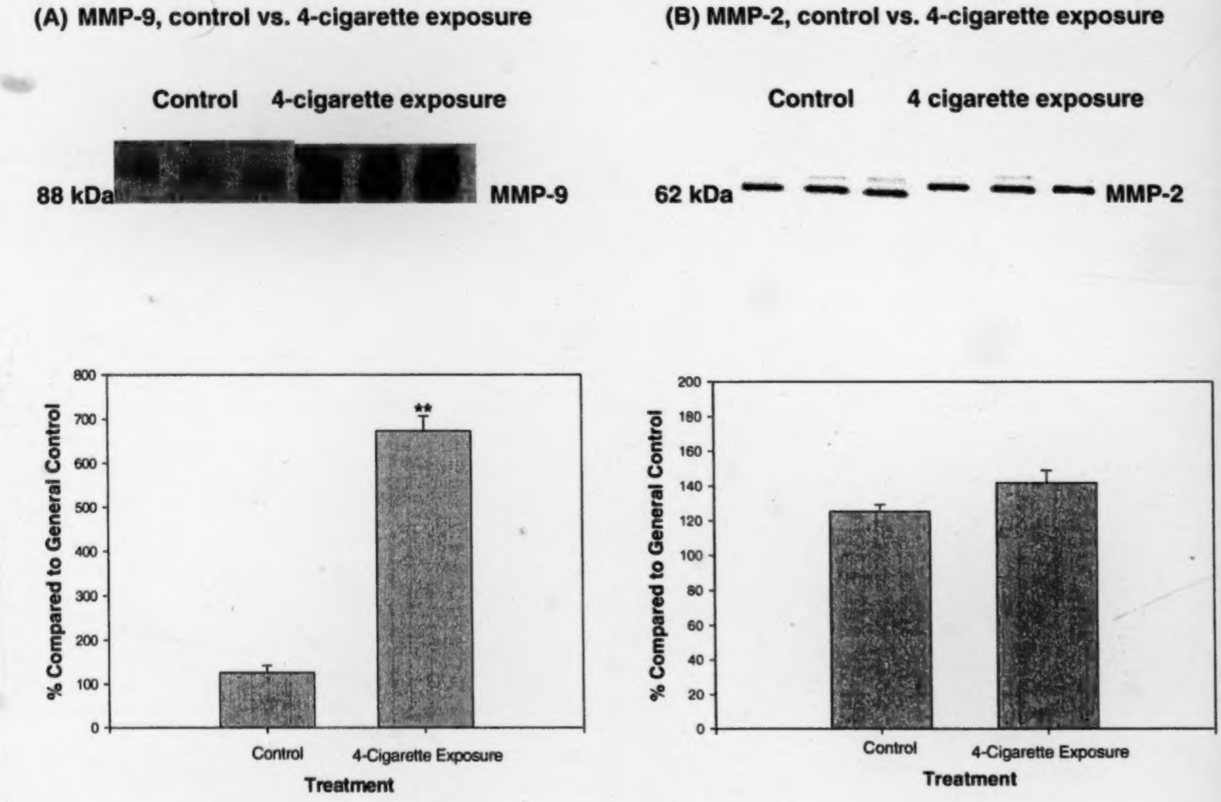


Figure 4.2 Comparison between normal and 4 cigarette smoke injured epithelial surface for mucociliary clearance function.

Mucociliary clearance time (MCT) from frog palates of control (n=3) and 4 cigarette smoke exposure (n=3). The data are presented as means ±SEM of the MCT. **p< 0.001 after 4 cigarette smoke exposure compared to control for MCT.



p<0.001, 546±49%, n=3

p=0.096, n=3

Figure 4.3 Comparison of MMP-9 and MMP-2 activation between normal and 4 cigarette smoke (CS) injured tissue.

Zymography analysis of MMP-9 activation from frog palate tissue (n=3, control; n=3, 4-cigarette smoke exposure; **A**). Also shown is MMP-2 activation from frog palate tissue (n=3, control; n=3, 4-cigarette smoke exposure; **B**). The upper panels show the zymograms (5 μg of protein per lane) for MMP-9 (**A**) and MMP (**B**). The lower panels show summary data as percent compared to the general control (5μg of protein from the untreated tissue). The data are presented as means ± SEM of percent compared to the control. ** p< 0.001 for MMP-9 activation from palate tissue after 4-cigarette smoke exposure compared to control.

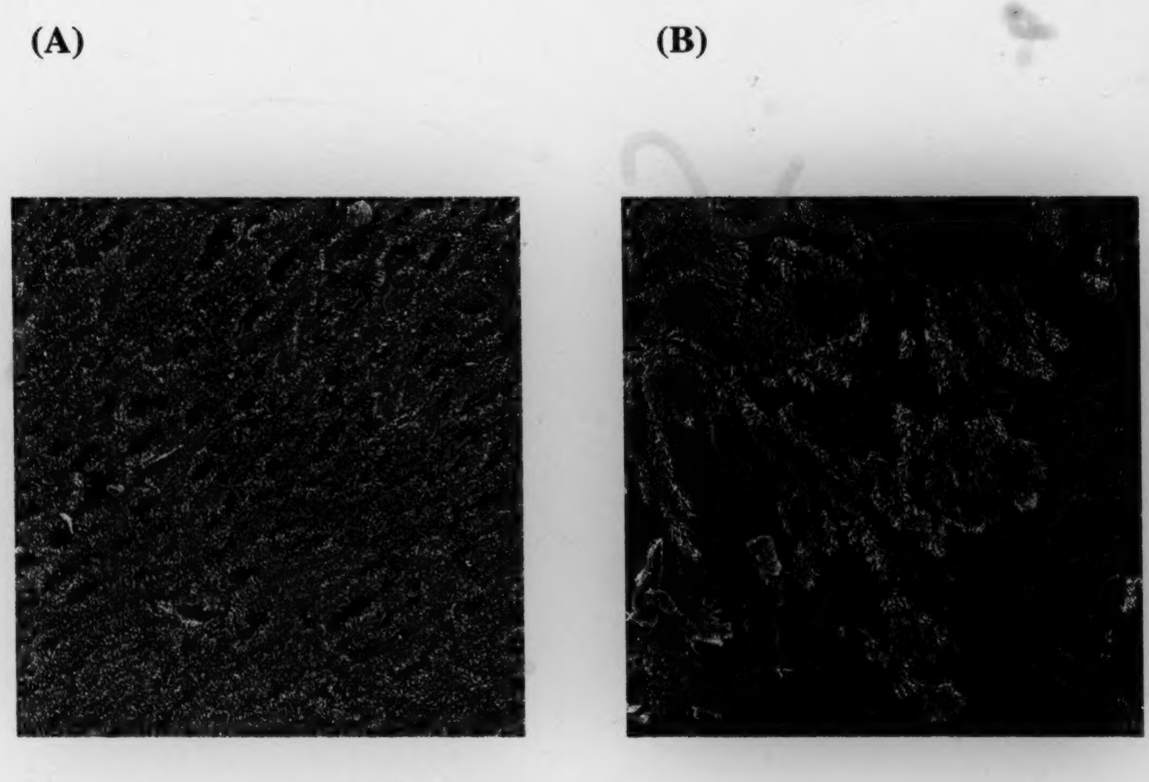


Figure 4.4 Scanning electron micrographs (SEM) of the epithelial surface (×3500) of normal frog palates and palate after 4-cigarette smoke exposure.

Figure 4.4A shows the tissue collected from the control half of the palate. The cilia appear as uniform fine dots covering the entire surface of the palate, interspersed with pores. **Figure 4.4B** shows the tissue collected from the half of the palate exposed to the smoke from four cigarettes. Large bare areas were detected, indicating a substantial loss of ciliated epithelial cells.

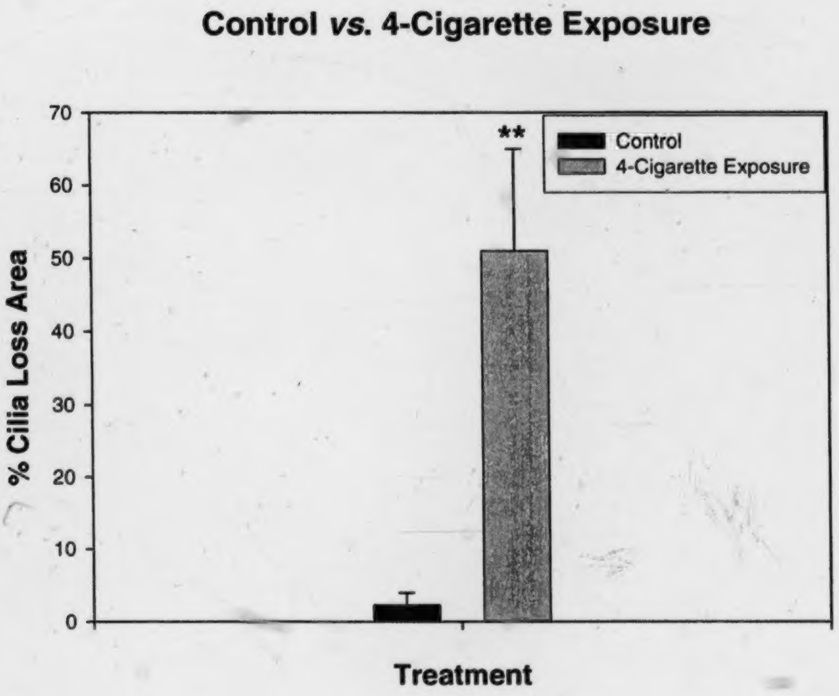


Figure 4.5 Comparison of cilia loss area between the control frog palate tissue and the tissue after 4-cigaette smoke exposure.

Areas of cilia loss were measured from acquired SEM images. Percent of cilia loss area was compared to the whole SEM area for control tissues (n=3) and for tissues after 4-cigarette smoke exposure. ** $p< 0.001$ for the difference in cilia loss area for the 4-cigarette smoke exposed tissue compared to control.

CHAPTER 5. INVOLVEMENT OF NITRIC OXIDE IN MMP-9 RELEASE FOLLOWING SODIUM METABISULPHITE ADMINISTRATION

5.1 INTRODUCTION

Nitric oxide (NO) is a well-known toxic agent, being a constituent of air pollution and cigarette smoke. In addition to being an environmental toxin, endogenously formed NO is thought to promote a number of chronic inflammatory diseases. Yet, at the same time, NO plays an important role in regulating several biological functions in the airway and non-specific host defence, and has antimicrobial activity against a wide variety of pathogens (1,2). It is known, for example that, in the presence of molecular oxygen (O_2), NO can form reactive nitrogen oxide species that can damage DNA, inhibit a variety of enzymes, and initiate lipid peroxidation (3).

A blueprint by which one may distinguish the regulatory processes and/or anti-inflammatory effects of NO from its potential toxic and/or proinflammatory properties is shown in **Figure 5.1** (4). Although NO possesses a wide array of regulatory and protective functions in inflammatory processes and has been shown to downregulate inflammatory cytokine production through the inhibition of NF-κB activation (11), active inflammatory conditions promote the oxidative metabolism of NO to reactive nitrogen species (RNS), which can affect biochemical pathways. NO production is a stress response and can lead to either tissue injury because of its radical chemistry, or be cytoprotective, protecting cells from damage by destroying pathogenic microorganisms.

Over-expression of NO or its reactive nitrogen species, ONOO, may promote tissue injury (enterocyte apoptosis) and inhibit tissue repair mechanisms (epithelial restitution via enterocyte migration and proliferation).

Epithelial cells express an inducible NO synthase (iNOS) in response to proinflammatory cytokines and in response to oxidants. The iNOS has much greater capacity to produce NO than the constitutive forms; it promotes oxidative reactions and is responsible for tissue injury. Inflammatory conditions of the respiratory tract are commonly characterized by elevated production of NO through increased expression of iNOS within the respiratory epithelium and in inflammatory cells, such as monocytes/macrophages and neutrophils (9, 10, 11). NO is detectable in the exhaled breath of animals and humans (5), and its concentration is increased in patients with asthma (6, 7) and with bronchiectasis (8).

Inhibition of NO synthase (NOS) has proved beneficial in treating conditions involving excessive NO production inducing tissue injury. Investigators have reported the effect of iNOS inhibitor on intestinal mucosal barrier function in a burn injury model. The decrease in NO production resulted in decreased formation of peroxynitrite and subsequently decreased damage of mucosal tissue (9). Numata et al. (21) also reported that inhibition of iNOS prevents lipopolysaccharide (LPS) -induced acute lung injury in dogs. N^G-Nitro-L-arginine methyl ester hydrochloride (L-NAME), a nonselective NO synthase inhibitor, is widely used in research studies requiring the inhibition of NO-induced tissue injury.

A number of recent studies have identified various regulatory properties of NO and/or reactive nitrogen species (RNS) on gene expression and activation of MMPs. For instance, several RNS are capable of activating MMPs, including human neutrophil collagenase (MMP-8) and MMP-9 (14, 15), via oxidative modification of the cysteine switch. In addition, NO has been reported to induce MMP-9 protein expression in chondrocytes (16) but was found to downregulate MMP-9 protein expression in mesangial cells (17) and aortic smooth muscle cells (18,19). However it was often unclear whether NO itself or RNS were responsible for these effects, and variable metabolism of NO in these different experimental designs may have yielded disparate effects on MMP expression and activation. Moreover, the potential effects of NO on MMP expression and activation in epithelial cells have not been studied (20). In our study, we investigated the role that NO played in the mucociliary clearance function and MMP expression and activation in the sodium metabisulphite (MB) injured frog palate epithelium.

5.2 MATERIAL AND METHODS

5.2.1 Animals and Experimental protocol

As previously described, from a bullfrog, *Rana catesbiana*, the upper portion of the head was removed, modifying the procedures described in previous work (26, 31), by cutting with scissors through from the junction of the posterior pharynx and esophagus out to the skin of the back. The excised palate was then inverted and placed immediately onto a petri dish with a piece of gauze soaked in FR. The palate was placed in the middle of the mucociliary clearance observation chamber.

5.2.2 Mucociliary clearance observation

Mucociliary clearance observation chamber

After the frog palate was isolated, it was placed in the middle of an acrylic chamber (see **Figure 2.1**, 20cm height, 30cm width, 20cm depth) maintained at a constant temperature (22°C to 24°C), which was continuously humidified by nebulization of FR solution. Mucociliary clearance movement was detected through a dissecting microscope placed above the chamber.

MCT measurements

As described in Chapter 2, normal mucociliary clearance time (MCT) was measured by applying a droplet of FR to the frog palate. The volume of the FR applied was proportional to the total epithelial area of this palate. Usually the volume of the solution applied ranged from 2μl to 5μl. Two minutes was allowed to elapse for the solution to be distributed over the palate before measurements. A drop of clear mucus (1-2mm) was then colleted from the palate and was placed on the top of the palate (near the mouth side). Once the mucus transport reached a steady rate, MCT was determined by recording the time for the displacement of mucus for 5mm. At least 5 MCT measurements were recorded to unite at a mean MCT after each treatment.

5.2.3 Frog palate exposure model preparation

Animal preparation

The study involved three groups:

In the control group (n=3), after the fresh frog palate was placed in the mucociliary clearance observation chamber and stabilized for 20 minutes, frog ringers solution (FR) was applied to the palate and MCT was measured and tissue samples were taken.

In the MB (10^{-1}M) injured group (n=3), after stabilization, FR was applied to the palate and left for 5 min. This was followed by the application of MB (10^{-1}M), then MCT was measured and tissue samples were taken.

In the L-NAME pre-treated MB (10^{-1}M) injured group (n=3), after the stabilization, L-NAME (CalBiochem-Nova Biochem Corp, La Jolla, Calif), made up in FR (10^{-4}M) was applied to the palate and left for five minutes. This was followed by the application of MB (10^{-1}M), followed by the measurement of mucus clearance function and then samples of palate tissue were taken.

5.2.4 Tissue collection

After the applications were completed, the epithelial tissue was carefully separated from the palate musculature. A piece of tissue (about 5mm × 15mm) was sectioned from the central part of each half palate, frozen in liquid nitrogen immediately and stored at –80°C for zymography studies.

5.2.5 Gelatin zymography

Tissue samples were collected as described above and protein concentrations were measured. The MMP-2 and MMP-9 activation in the epithelial tissues were evaluated by zymography according to established methods as described in Chapter 3 and 4 (13, 15, 25, 27, 32, 33, 34, 35, 36).

We detected two bands at the site of 62 kDa and 68 kDa of MMP-2 and a band at the site of 88kDa of MMP-9 in our zymographies. MMP-2 and MMP-9 were identified and detected as described in Chapter 3 and 4.

5.2.6 Statistics

One way ANOVA was used to compare among three groups. Significance was considered when $p < 0.05$.

5.3 RESULTS

5.3.1 The effects of L-NAME (10^{-4}M) on mucociliary clearance function in the MB (10^{-1}M) injured palate

One way ANOVA confirmed significant differences of MCT among the three groups of different treatments ($p<0.001$, n=3). In the group of frog palates injured by MB (10^{-1}M), MCT was significantly increased by (~100%, $p<0.001$, n=3) compared to the control group (**Figure 5.2**). In the group of frog palate pre-treated by L-NAME (10^{-4}M) and injured by MB (10^{-1}M), MCT was significantly decreased (~90%, $p<0.01$, n=3) compared to the MB (10^{-1}M) injured group (**Figure 5.2**) and was not changed appreciably ($p=0.27$, n=3) compared to the control group (**Figure 5.2**).

5.3.2 The effects of L-NAME (10^{-4}M) on MMP activation in the MB (10^{-1}M) injured tissue

The zymography result of the tissues taken from the fresh untreated frog palate (n=3) was used as a general control.

We carried out zymographic analyses comparing activation of MMP-9 and MMP-2 from tissues collected from the control group (n=3), MB (10^{-1}M) injured group (n=3) and L-NAME (10^{-4}M) pre-treated MB (10^{-1}M) group (n=3) of the palate (**Figure 5.3**).

We detected MMP-2 (62kDa, 68kDa) and MMP-9 (88kDa) in the supernatants of the tissue samples. One way ANOVA confirmed significant difference for the activation of MMP-9 among the three groups of different treatments ($p<0.001$, $n=3$). Once again we found significantly increased activation of MMP-9 from the tissues collected from the MB (10^{-1}M) injured palates compared to the control (610±31%, $p<0.001$, $n=3$; **Figure 5.3**). The data demonstrated that tissue collected from the L-NAME (10^{-4}M) pre-treated, MB (10^{-1}M) injured palates has significantly less MMP-9 activation compared to the non-L-NAME-treated group (537±36%, $p<0.001$, $n=3$; **Figure 5.3**). As before, there was little effect on MMP-2 activation with MB (10^{-1}M) treatment, and pre-treatment with L-NAME (10^{-4}M) did not affect the appearance of MMP-2.

5.4 DISCUSSION

Nitric oxide is produced by nitric oxide synthase (NOS) from L-arginine, with molecular oxygen as the substrate. NOS is known to have three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). In order to investigate the roles of NO such as versatile bioactivities and the cytotoxicities of the metabolites, it is important to impede the enzymatic NO production by NOS inhibitors. As L-arginine is a substrate of NOS, a large number of L-arginine analogs, such as L-NMMA and L-NAME, have been developed as NOS inhibitors. They are indispensable tools for the study of nitric oxide related biological processes. L-NAME is an analog of L-arginine methyl ester, which has a nitro group on the N^G of the guanidino moiety. It is widely used as a nonselective NOS inhibitor (30). Since we are not sure which kind(s) of NOS are involved in the MB induced tissue injury, we chose L-NAME in our experiment instead of other NOS inhibitors because of its nonselectivity.

MB, which releases SO_2, can cause inflammatory effects to the epithelium (22). The iNOS expressed by epithelial cells in response to the proinflammatory cytokines released by the inflammatory cells or epithelial cell has a much greater capacity to produce NO than the constitutive forms, promotes oxidative reactions and is responsible for tissue injury. The results of our study have demonstrated that the group of frog palates injured by MB (10^{-1}M) but pre-treated by L-NAME maintained a less disturbed mucociliary clearance function than the non-L-NAME treated injury group (**Figure 5.2B**). This suggested that the NO may be involved in the frog palate epithelial injury induced by MB and inhibition of NOS, thus inhibition of synthesis of NO, may have protective effects on the tissue injury caused by MB induced inflammation.

The MMP family of enzymes are important in tissue development and regeneration, and inappropriate MMP regulation and activation during inflammation, may lead to disturbances in the turnover and remodeling of the pulmonary extracellular matrix, which could contribute to structurally inappropriate remodeling. In particular, MMP-2 and MMP-9 possess type IV collagenolytic activity, which is important in the destruction/repair of the epithelial basement membrane in the lung. Whereas MMP-2 is constitutively expressed in various cell types, MMP-9 is strongly induced in epithelial cells by inflammatory cytokines, particularly TNF-α (23, 24). Inflammatory conditions are also associated with increased activation of oxidant-producing enzymes and NO, which could directly affect MMP expression or activation (14, 15, 16, 28).

In the zymography experiment, we found that the MB-injured group pre-treated by L-NAME released significantly less MMP-9 activation than the MB-injured group with no L-NAME treatment (**Figure 5.3**). The downregulation of MMP-9 activation by L-NAME in the MB injured frog epithelial tissue is an indication of the important involvement of NO in the upregulation of MMP-9 activation in the MB epithelial injury. No significant MMP-2 activation changes were found in MB injured palates treated with or without L-NAME. It has been reported that excessive NO can cause tissue injury (29). In our study, the results have supported our hypothesis that NO-caused tissue injury may be one of the possible mechanisms of the injury induced by MB. However, it is still not clear that whether NO itself or RNS were responsible for the injury. Furthermore, various metabolites of NO may be produced in the process of the inflammation and injury; whether the upregulation of MMP-9 activation is due to NO itself or its metabolites still needs to be investigated. However, using superoxide dismutase to remove O^{2-} and prevent $ONOO^-$ formation would narrow possible NO metabolites. Further investigations need to be done.

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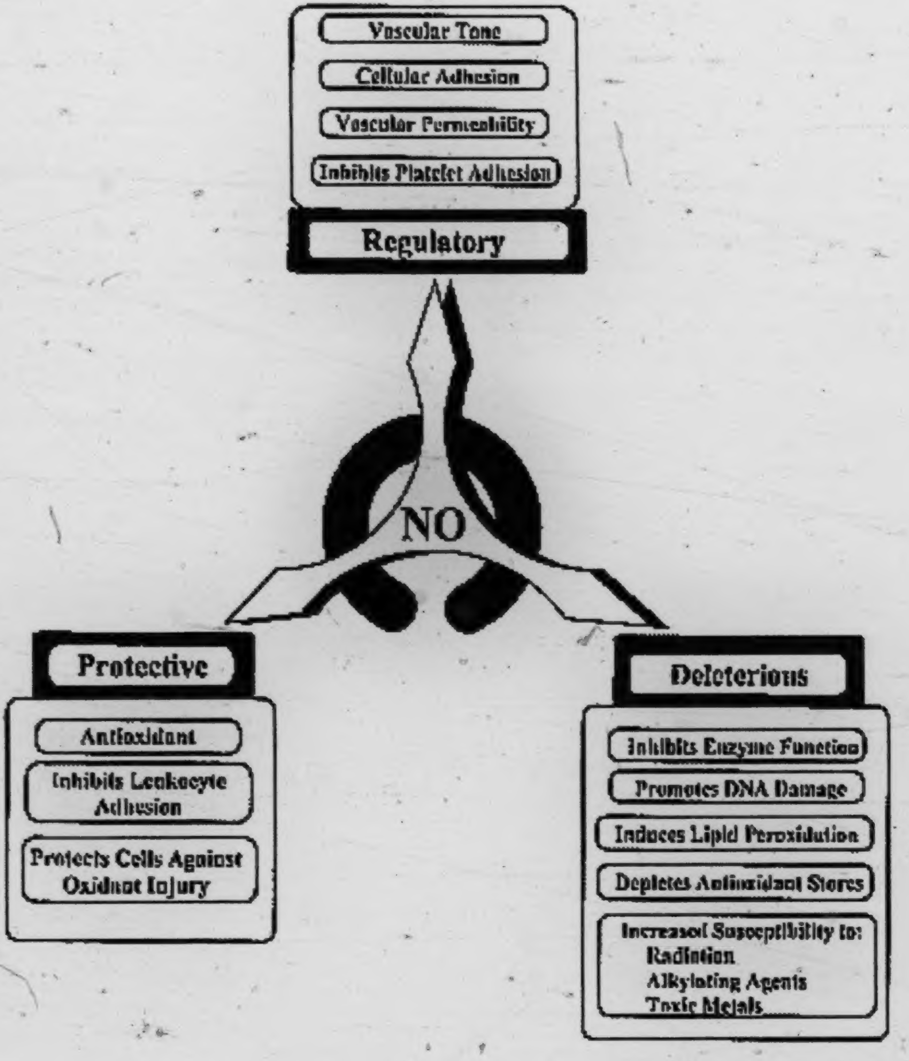

Figure 5.1 Regulatory, protective and deleterious biological effects of nitric oxide (NO).

Comparison of Mucociliary Clearance Time, Control vs. MB (10^{-1}M) With&Without L-NAME Pre-treatment

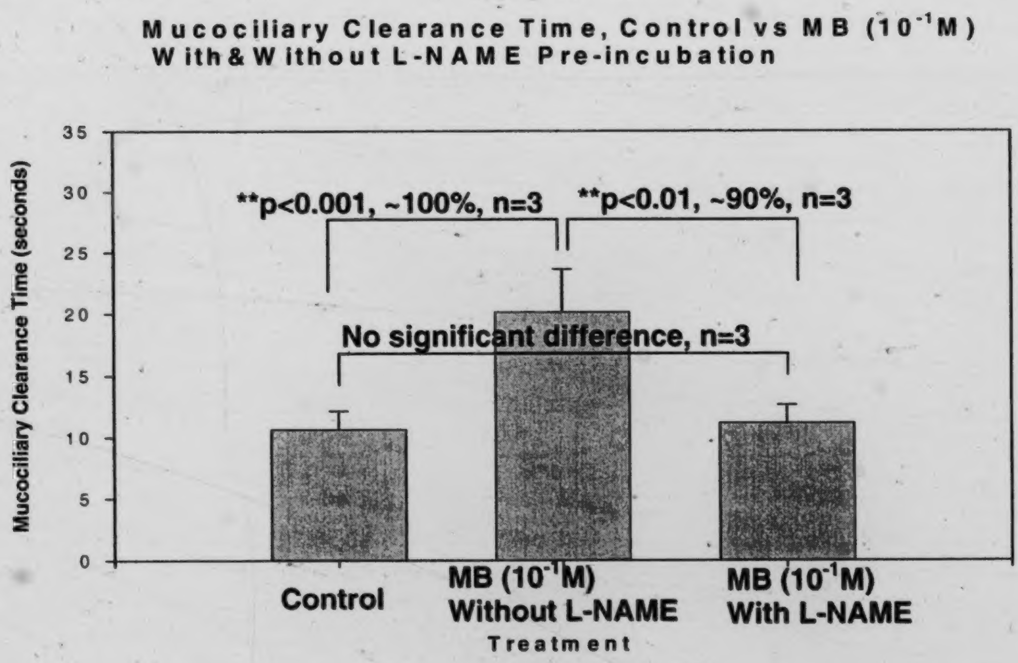

Figure 5.2 The effect of L-NAME (10^{-4}M) pre-treatment on mucociliary clearance function for MB injured palate.

Mucociliary clearance time (MCT) of the frog palates (n=3, control; n=3, MB (10^{-1}M) treated without L-NAME (10^{-4}M) pre-treatment; n=3, MB (10^{-1}M)). The data are presented as means ±SEM of the MCT. ** $p<0.001$ at MB (10^{-1}M) (without L-NAME) treated palate compared to control for MCT. ** $p< 0.01$ at the MB (10^{-1}M) treated palate with L-NAME (10^{-4}M) pre-treatment compared to without L-NAME (10^{-4}M) pre-treatment for MCT.

Comparison of MMP-9 Expression, Control vs. MB (10^{-1}M) With&Without L-NAME Pre-treatment

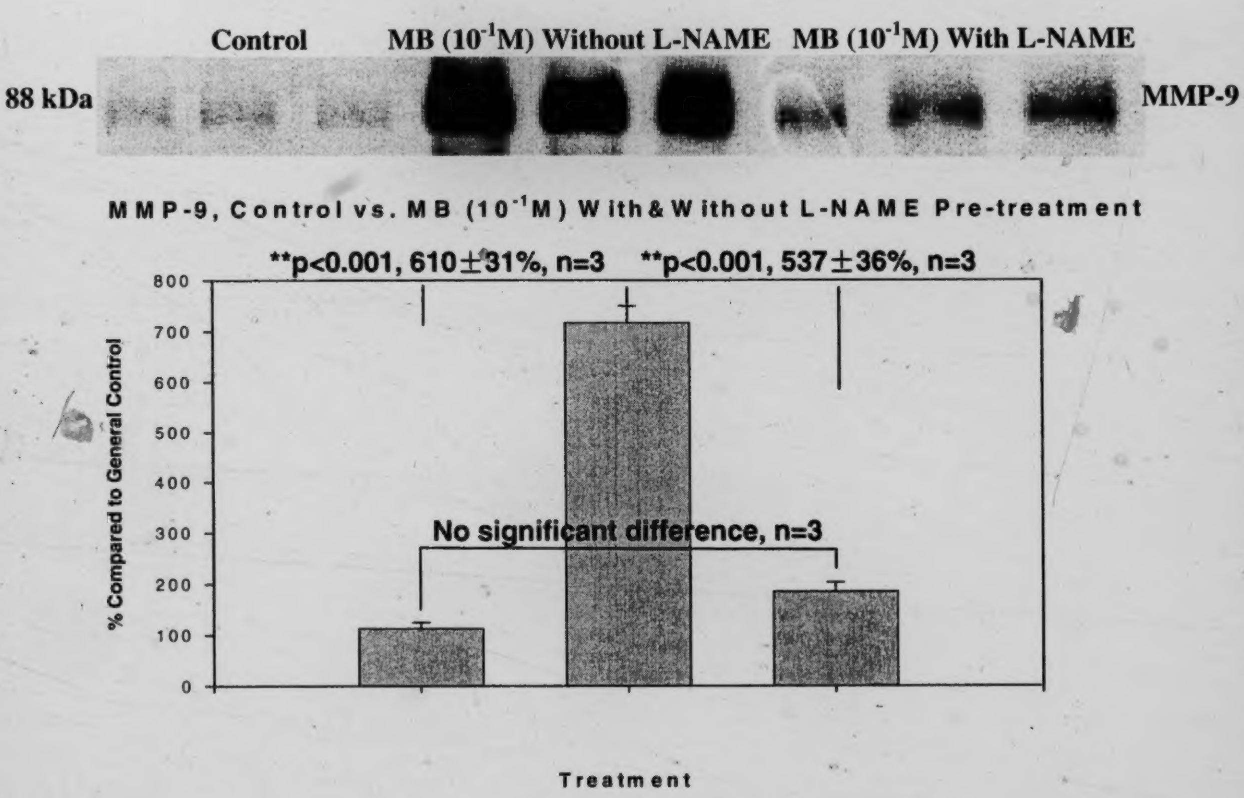

Figure 5.3 Comparison of MMP-9activation among tissues from normal frog palate and from sodium metabisulphite (MB) injured frog palate with/without L-NAME (10^{-4}M) pre-treatment.

Zymography analysis of MMP-9 activation from frog palate tissue (n=3, control; n=3, MB (10^{-1}M); n=3, L-NAME+ MB (10^{-1}M)). The upper panels show the zymogram (5 μg of protein per lane) of MMP-9 activation. The lower panels show summary data as percent compared to the general control (5μg of protein from the untreated tissue). The data are presented as means ± SEM of percent compared to the control. ** $p < 0.001$ for MMP-9 activation of MB (10^{-1}M) treated tissues. ** $p < 0.001$ for MMP-9 activation of L-NAME pre-treated injured tissue compared to the injured tissue without pre-treatment.

CHAPTER 6. GENERAL DISCUSSION

6.1 SUMMARY

The major focus of this thesis has been the study of the role of matrix metalloproteinases (MMPs) in sodium metabisulphite (MB)- and cigarette smoke (CS)-induced frog palate epithelial injury, specifically with respect to tissue remodeling and epithelial reactivity. The protective effects of ophiopogonis (OP) in MB injury and the involvement of nitric oxide (NO) were also studied. These aspects were studied on the bullfrog (*Rana Catesbiana*) model.

Exposure to MB released sulphur dioxide (SO_2), and to cigarette smoke induces tissue injury and mucociliary clearance dysfunction. The intricacies of tissue remodeling, however, remain incompletely understood. Using tissues collected from normal healthy frog palate models and those injured by MB, we demonstrated an increased activation of MMP-9 and a decreased release in a protein, consistent with TIMP-1 in the injured tissue. Mucociliary clearance dysfunction was also detected in MB released SO_2 and CS exposure. These results suggest that acute exposure to high concentrations of MB or CS induce tissue injury and remodeling as well. Similarly, tissues collected from frog palates exposed to CS demonstrated increased activation of MMP-9 compared to tissues from normal palates, suggesting that acute exposure to CS can also induce tissue injury and remodeling. The observation of mucociliary clearance dysfunction suggested that cilia were seriously affected during this process.

We also investigated whether frog palates pre-incubated in OP demonstrated alterations in mucociliary clearance and activation of MMP and the release of TIMP-1-like protein compared to palates pre-incubated in frog ringer (FR), and whether this behaviour may be affected by the dose of MB applied. Indeed, alterations in mucociliary clearance induced by MB were significantly attenuated by OP, and the observed levels of MMP-9activation and TIMP-1-like protein were partially normalized. Finally, we studied one of the possible pathways of MB-induced frog palate tissue injury. We demonstrated that nitric oxide (NO) was involved in mediating epithelial reactivity, specifically acting as a proinflammatory agent in MB-induced epithelial tissue injury and regulating the MMPs. Using tissues pre-treated with the iNOS inhibitor L-NAME and injured by MB, we demonstrated reduced activation of MMP-9 compared to those tissues without this treatment and injured by MB, suggesting that tissue injury caused by NO may be one of the possible mechanisms for the induction of tissue injury by MB.

6.2 THE FROG PALATE MODEL

The frog palate has a pseudostratified epithelium composed of both ciliated and mucus-secreting cells and covered with a two-layered periciliary film; since this is quite similar to the situation in human conductive airways, and because of the difficulties in studying mucociliary clearance in intact mammalian airways, the frog palate has been used for several decades as a model to assess mucociliary clearance (7, 8, 9, 10). Different species of frogs have been used, as well as a variety of study designs. Investigators had to deal with several sources of variability, making it difficult to standardize a widely acceptable model. There are disadvantages in this model such as: it pertains a non-mammalian species, and in addition, the epithelial tissue is non-respiratory. Moreover, it is a model that not widely used in other research fields, so that the monoclonal antibodies are usually not available commercially, which led to our difficulties in western blots. However, as a model it has advantages over some mammalian models, such as rodents, in that the ciliated epithelium has a well-developed mucus blanket that works in coordination with cilia similar to the human situation. We performed an *ex-vivo* study on the frog palate model, which disabled the circulation. This may have impaired the studies on the recruited inflammatory cells after the MB and CS injury, but it also focused the study on the oral epithelial cells. In our model, some released mediators, toxic reagents or other chemicals are more likely from the epithelial cells rather than the inflammatory cells.

6.3 MUCOCILIARY CLEARANCE DYSFUNCTION OF THE INJURED EPITHELIUM

Mucociliary clearance function is critical to pulmonary defence (1). Mucociliary clearance time (MCT) is one of the mostly used measurements of the function of the mucociliary system (2). It is the time recorded by measuring the rate of displacement of a certain distance with a certain mucociliary clearance velocity (MCV) (2). We hypothesized that delayed MCT, which indicates mucociliary dysfunction, would be detected in frog palates injured by MB or CS. The results of our experiments were consistent with this hypothesis, thus suggesting, in part, that the injury induced by MB and CS can disrupt the normal cilia physiology and/or performance. These observations are supported by recent studies that show cilia loss in MB injured frog palate (4) and decreased cilia beating frequency in CS exposed bovine lung cells (5).

In this study we used MCT as an indicator of mucociliary clearance function in the airways. Although cilia disruption always leads to delaying of the MCT, and we usually can indicate the mucociliary clearance function according to the magnitude of MCT, our work would be stronger if supported by the direct observations of cilia action. Because of technical limitations, we were unable to make an assessment of ciliary beat frequency (CBF). Thus, the action of the cilia, particularly, the effect of MB or CS on CBF was not measured. The initial acute effect of MB and CS may have been a transient effect on CBF resulting in a marked slowing of MCT. Secondly, although we measured MCT, we did not attempt to measure secretion rates. Future studies using the frog palate injury model will include a measurement of secretion rates following MB and CS treatments to the palate to answer the question of whether or not MB and CS stimulate hypersecretion in this model.

6.4 ALTERED ACTIVATION OF MMPS AND RELEASE OF THEIR INHIBITORS-LIKE PROTEINS IN INJURED FORG PALATE EPITHELIUM INDUCED BY SODIUM METABISULPHITE AND CIGARETTE SMOKE

MMPs are critical mediators of tissue injury and remodeling in various physiological and pathophysiological processes. We hypothesized that higher levels of MMP-9 would be released from the frog palate injured by MB and CS. The results of our study were consistent with the hypothesis. Interestingly, increased activation of MMP-9 was observed in both MB (10^{-2}M) and (10^{-1}M) applications while there was almost no delay of MCT observed in MB (10^{-2}M) injured palate. These observations suggest that MMP-9 may play a very important role in the tissue injury and remodeling and it may be one of the mediators that can lead to further physiological disruptions; and MCT may not be as sensitive a measurement of functional change as MMP measurement.

In this study we planned to use MMP and TIMP secretory profiles as indicators of tissue injury and remodeling in the frog palate epithelium. However, since little research work has been done on frog tissue, the proteins we detected can not be identified as frog TIMP-1. Further investigations need to be done to identify these proteins in frog tissue. Our work would have benefited from the provision of anti-frog MMP antibodies.

6.5 THE ROLE MMPS PLAY IN INJURED TISSUE REMODELING

MMPs are not expressed in normal, healthy, resting tissues; at least their production and activity are maintained at nearly undetectable levels. In contrast, in any diseased or inflamed tissue, some level of MMP expression or upregulation is seen. Though the qualitative pattern and quantitative levels of MMPs vary among diseases, injuries, tumor types, inflammatory conditions, and cell lines, a reasonably safe generalization is that activated cells express increased amount of MMPs. Before we can understand the regulation and function of MMPs, we need to know where, when, and by which cells these proteinases are produced. In our study, we used an ex-vivo frog palate model, in which there is no circulation. In addition, the tissue was removed frog the palate no more than ten minutes after application. Under this situation, inflammatory cells are less likely to be the major source MMP-9. However, epithelial cells also can release MMP-9 when activated. Since we detected increased activation of MMP-9 in the tissues injured by MB and CS, and since inflammatory cells are almost excluded from the major sources of MMP-9, this suggests that activated epithelial cells may be one of the sources of the MMP-9 we detected in our studies.

The TIMPs are a family of polypeptides that form noncovalent complexes with either active or latent MMPs and inhibit their activity and/or activation. All the TIMPs appear to have activities against all members of MMPs family. TIMP-1 is secreted with MMP-9; it influences MMP-9 processing during activation and affects its activity, as well. Airway remodeling is regulated, at least in part, by the interplay between MMP-9 and TIMP-1. Recent studies have shown that in sputum of asthmatic and chronic bronchitis subjects, the levels of TIMP-1 are significantly increased compared with those of control subjects (13), and the molar ratio between MMP-9 and TIMP-1 is significantly lower than in control subjects, suggesting the existence of a protease-antiprotease imbalance. It is important to note that in different models, different cell types, different levels of MMPs and TIMPs are expressed. In our study, in the frog epithelial tissue, high activation of MMP-9 was detected in the injured tissue compared to the control; however, the injured tissues were also demonstrated to have lower TIMP-1-like protein levels compared to the control. If we could identify TIMP-1-like protein as frog TIMP-1, this study would demonstrate that injury by MB and CS may lead to a higher molar ratio between MMP-9 and TIMP-1, suggesting the existence of a protease-antiprotease imbalance.

6.6 OPHIOPOGONIS MEDIATED MUCOCILIARY CLEARANCE FUNCTION AND MMP/TIMP ALTERATION IN SODIUM METABISULPHITE INJURED FROG PALATE SURFACE AND TISSUE

We hypothesized that the herb Ophiopogonis (OP) has a protective effect on the injury induced by MB. In this study, we demonstrated that the frog palates pre-incubated in OP decoction overnight had a better ability to maintain the mucociliary clearance function under the injury caused by MB compared to the palates pre-incubated in frog ringer (FR) as the controls. This protective effect of OP may due to its protection of the cilia, since we observed a healthy and almost intact cilia bed in the SEM studies of the OP pre-incubated injured palates. Furthermore, it has also been demonstrated that tissues from OP pre-incubated MB injured palates showed significantly less MMP-9 activation than FR-incubated MB injured palates, this suggested that OP may partially inhibit the imbalance of protease-antiprotease.

As a traditional Chinese herbal medicine used for treating obstructive respiratory diseases, the mechanism of the action of OP on the airways and the pathway of its effects still remained unclear. It is also difficult to demonstrate its specific effect under normal conditions, as the most of the beneficial effects of traditional herbal medications have been shown in pathologic states. Therefore our injury model may be particularly suited for the study of OP. Further investigations will be carried to detect more information about OP and its protective or restorative actions.

6.7 THE ROLE OF NITRIC OXIDE IN SODIUM METABISULPHITE INDUCED EPITHELIAL INJURY

In physiologic states, NO can serve a protective function, but under conditions of high output, it is toxic. The direct toxicity of nitric oxide is modest but is greatly enhanced by reacting with superoxide to form peroxynitrite (ONOO-), a strong oxidant (14, 15), which may contribute to tissue damage. In contrast, NO can also blunt the inflammatory response via its ability to inhibit NF-κB activation by increasing the expression, nuclear translocation, and stabilization of its inhibitory protein IκB (16, 17). However, these effects are not straightforward, as some studies have suggested that NO can increase the expression of some inflammatory response proteins such as cyclooxygenase-2 (COX-2), TNF-α, and iNOS (18, 19). It has been suggested that NO may exert both deleterious and protective effects in sepsis by regulating NF-κB either positively or negatively, depending on species, timing, the cell type, inflammatory stimulus, the NO concentration, and NO-related metabolites generated (20). Alveolar macrophages and airway epithelial cells were demonstrated to be major cells in which cytokines induce iNOS expression and NO formation, although endothelial cells can also be induced to express iNOS (21, 22). The precise roles of NO in MB and CS induced inflammation and frog palate tissue injury are still under debate, because it is beneficial in certain conditions but it also can have toxic effects (3, 23, 24). This is reflected in the conflicting reports regarding the effects of NOS inhibition or knockout on inflammation. Part of the difficulty in assigning a precise role to NO is the dual nature of this important mediator (12).

In our study we demonstrated that the pre-treatment of non-selective NOS inhibitor L-NAME had protective effects in the mucociliary clearance function of the frog palates injured by MB. We also found that the L-NAME pre-treated injured group showed significantly less MMP-9 activation as well. It seemed that the MB-induced injury may depend on or at least partially depend on NO synthase modulation. Based on these findings, NO may be one of the major mediators leading to tissue injury in this case. However, there also may be beneficial effects of NO in this process; this aspect needs to be investigated in the future.

6.8 SIGNIFICANCE OF EXPERIMENTS

This thesis presents novel information on MB and CS- injured frog palate models. MB releases SO_2 when it dissolves in water. Investigators have reported the airway tissue inflammation and injury to mammalian airways when chronically exposed to SO_2 in the polluted air (6, 11). However, little is known when airways are acutely exposed to high concentration of SO_2, which may happen during accidental occupational exposure to SO_2. In our study, we applied MB (10^{-1}M) to the frog palate, which can generate the amount of SO_2 more than that in the polluted air. Since the frog palate shares many features in common with the mammalian airways, this study may provide some useful information to facilitate studies of mammalian airways undergoing same injury. We have demonstrated impaired mucociliary clearance function, increased activation of MMP-9, and decreased TIMP-1-like protein in injured tissues compared with normal tissue.

OP pre-incubation of the MB injured frog palates mediated an MMP/TIMP alteration and provided protection for mucociliary clearance function compared to FR pre-incubated injured palates; these findings suggest one possible way to justify the clinical use of this traditional herbal medicine. Furthermore, the studies examining the role of MMPs and the mucociliary clearance function in epithelia acutely injured by cigarette smoke should add to our understanding of the pathophysiology of short-term, high concentration second-hand smoke exposure. The possible role played by NO in the MB induced injury was also investigated; these studies have opened the doors to an intriguing tissue injury pathway induced by MB. Future research in these areas will expand our understanding of the roles of MMPs in the tissue injury and remodeling and the clinical effect and mechanism of herbal drugs such as OP.

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